

WATER SOLUBLE VITAMIN STATUS OF LOW
BIRTHWEIGHT INFANTS RECEIVING
TOTAL PARENTERAL NUTRITION

CENTRE FOR NEWFOUNDLAND STUDIES

**TOTAL OF 10 PAGES ONLY
MAY BE XEROXED**

(Without Author's Permission)

RYNA LEVY



**Water Soluble Vitamin Status
of Low Birthweight Infants Receiving
Total Parenteral Nutrition**

A Thesis presented to
the Department of Biochemistry
of
Memorial University of Newfoundland

by

Ryna Levy

In partial fulfillment of the
requirements for the degree of
Masters of Science

January, 1990

© R. Levy 1990



National Library
of Canada

Bibliothèque nationale
du Canada

Canadian Theses Service Service des thèses canadiennes

Ottawa, Canada
K1A 0N4

The author has granted an irrevocable non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of his/her thesis by any means and in any form or format, making this thesis available to interested persons.

The author retains ownership of the copyright in his/her thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without his/her permission.

L'auteur a accordé une licence irrévocable et non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de sa thèse de quelque manière et sous quelque forme que ce soit pour mettre des exemplaires de cette thèse à la disposition des personnes intéressées.

L'auteur conserve la propriété du droit d'auteur qui protège sa thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

ISBN 0-315-59219-2

DEDICATION

To Elsie and George Levy, my parents. Together they have provided me with much love, support and continuous encouragement.

ABBREVIATIONS

EGR (AC)	erythrocyte glutathione reductase (activity coefficient)
ETA	erythrocyte transketolase activity
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
FTM	full term human milk
GSSG	oxidized glutathione
GSH	reduced glutathione
IF	intrinsic factor
LBW	low birthweight
PDA	persistent ductus arteriosus
Pte-Glu	pteroyl polyglutamic acid
PTM	preterm human milk
RDS	respiratory distress syndrome
TPN	total parenteral nutrition
TPP (E)	thiamin pyrophosphate (effect)

ABSTRACT

Vitamin recommendations for low birthweight (LBW) infants on total parenteral nutrition (TPN) are met with 3 mL of a multivitamin supplement, MVI-Pediatric (McGraw Laboratories). However, there is limited data available on the adequacy of this preparation and on the exact needs of infants receiving TPN. The purpose of this study was to assess the thiamin, riboflavin, folate and vitamin B₁₂ status of parenterally fed LBW infants receiving MVI-Pediatric. Thirty infants were randomly assigned to receive either 3 mL of MVI Pediatric (PAR3 group; birthweight = 1133 ± 293 g; gestational age = 29.4 ± 2.1 weeks) or 2 mL (PAR2 group; 1137 ± 231 g; 28.5 ± 1.8 weeks). One hundred percent (%) received TPN for the first week, 50% by the second, and less than 33% by the third. Eighteen control infants received enteral feeds only (ENT group; 1281 ± 271 g; 29.6 ± 1.7 weeks).

Weekly blood, 24 hour urine collections and dietary intake data were obtained. The adequacy of thiamin and riboflavin was assessed by erythrocyte transketolase activity (ETA) and erythrocyte glutathione reductase activity coefficient (EGRAC) respectively. Urinary thiamin and riboflavin levels were measured by fluorometric techniques. Plasma folate, red blood cell (RBC) folate, urinary folate and plasma vitamin B₁₂ concentrations were determined by a radioassay. As well, the thiamin, folate and B₁₂ levels in the TPN admixture and MVI-Pediatric were analyzed.

The ETA and EGRA were within the normal range for all

groups at each time period. No significant differences were found in ETA among the 3 groups. The EGRAC did differ significantly between the PAR2 (0.98 ± 0.07) and PAR3 groups (1.11 ± 0.10) at week 2 but no difference was observed between the ENT group and the parenteral groups at any time. Urinary thiamin and riboflavin levels were significantly lower prior to the initiation of parenteral or enteral feeds than at subsequent sampling times. However, there was no difference found between groups during the study period.

No difference was observed in RBC folate levels at any time. Plasma folate did significantly differ between the PAR3 group (24 ± 7 ng/mL) and both the PAR2 (13 ± 5 ng/mL) and ENT groups (16 ± 3 ng/mL) prior to the initiation of feeds and at week 1 and between the PAR3 (30 ± 16 ng/mL) and PAR2 (16 ± 4 ng/mL) infants at week 2. A difference was found in plasma vitamin B₁₂ between the ENT groups (551 ± 287 pg/mL) and both the parenteral groups (PAR2 = 841 ± 405 pg/mL; PAR3 = 924 ± 424 pg/mL) at week 1 and between the ENT (530 ± 238 pg/mL) and PAR3 (999 ± 425 pg/mL) groups at week 2.

From the results of the present study it appears that 2 mL of MVI-Pediatric is sufficient to meet the thiamin, riboflavin, folate and vitamin B₁₂ needs of the parenterally fed LBW infant.

ACKNOWLEDGEMENTS

My sincere gratitude is extended to Dr. James K. Friel for his guidance and supervision throughout my program. I also gratefully acknowledge the other members of my supervisory committee for their help and assistance, Dr. G. Herzberg and Dr. W. Andrews. For forever providing technical assistance cheerfully and skillfully, a special thank you to Craig Skinner.

I would like to convey special thanks to some people who were actively engaged at various stages of the project - the nurses in the Neonatal Unit at the Grace General Hospital and in the Neonatal ICU and Nursery at the Janeway Child Health Center; the pharmacy and laboratory staff at the Janeway Child Health Center; and to Shirley Hood and Marg Ewtushik.

To my family members and friends, I give my warm appreciation for their constant support. I would like to especially thank Rick Nurse for his consistent guidance and ongoing editing of this thesis.

Lastly, but most importantly, I wish to extend my sincere thanks to all the parents and their infants who so kindly participated in this study, for without them, this investigation would not have been possible.

This research was funded by the Banting Research Foundation.

TABLE OF CONTENTS

	PAGE
ABBREVIATIONS	(i)
ABSTRACT	(ii)
ACKNOWLEDGEMENTS	(iv)
CHAPTER 1.0 INTRODUCTION	1
CHAPTER 2.0 LOW BIRTHWEIGHT (LBW) INFANTS	5
2.1 DEFINITION OF PREMATURITY	5
2.2 PHYSIOLOGICAL HANDICAPS ASSOCIATED WITH PREMATURITY	5
2.3 METHODS OF FEEDING	
2.3.1 Parenteral Nutrition	10
2.3.2 Gastric Enteral Feeding	14
2.3.3 Types of Milk Available for Enteral Feeding	15
2.3.3.1 Human Milk	15
2.3.3.2 Commercially Prepared Formula	19
CHAPTER 3.0 VITAMIN METABOLISM IN HUMANS	22
3.1 INTRODUCTION	22
3.2 THIAMIN METABOLISM	23
3.3 RIBOFLAVIN METABOLISM	29
3.4 FOLATE METABOLISM	39
3.5 VITAMIN B ₁₂ (COBALAMIN) METABOLISM) ...	54
CHAPTER 4.0 PROBLEM STATEMENT	63
CHAPTER 5.0 METHODS	66

5.1	INTRODUCTION	66
5.2	SUBJECTS	66
5.3	SAMPLE COLLECTION	68
5.3.1	Blood Collection	68
5.3.2	Urine Collection	69
5.4	VITAMIN ANALYSIS OF BLOOD SAMPLES	70
5.4.1	Thiamin	70
5.4.2	Riboflavin	74
5.4.3	Folate and Vitamin B ₁₂	77
5.5	VITAMIN ANALYSIS OF URINE SAMPLES	80
5.5.1	Thiamin	80
5.5.2	Riboflavin	82
5.5.3	Folate and Vitamin B ₁₂	83
5.6	DIETARY INTAKE DATA	85
5.7	PARENTERAL NUTRITION INFUSATES	85
5.8	STATISTICAL ANALYSIS	86
CHAPTER 6.0	RESULTS	88
6.1	INTRODUCTION	88
6.2	VITAMIN CONCENTRATIONS IN TPN ADMIXTURES AND MVI-PEDIATRIC	94
6.3	THIAMIN	96
6.4	RIBOFLAVIN	105
6.5	FOLATE	113
6.6	VITAMIN B ₁₂	123
CHAPTER 7.0	DISCUSSION	129
7.1	INTRODUCTION	129

7.2	VITAMIN CONCENTRATIONS IN THE TPN ADMIXTURE AND MVI- PEDIATRIC	130
7.3	THE BALANCE TECHNIQUE	134
7.4	THIAMIN	136
7.5	RIBOFLAVIN	140
7.6	FOLATE	145
7.7	VITAMIN B ₁₂	148
CHAPTER 8.0	CONCLUSION	151
REFERENCES	153
APPENDIX A	168
B	169
C	170
D	170
E	171
F	173
G	174
H	175
I	176
J	177
K	178

LIST OF TABLES

TABLE	PAGE
2.1 PHYSIOLOGICAL HANDICAPS OF THE LBW INFANT	6
2.2 SUGGESTED DAILY INTAKES OF VITAMINS AND TRACE MINERALS FOR LBW INFANTS RECEIVING TPN	13
2.3 COMPOSITION OF PTM AND FTM DURING THE FIRST MONTH OF LACTATION	17
2.4 NUTRIENT COMPOSITION OF PROPRIETARY INFANT FORMULAS FOR LBW BIRTHWEIGHT INFANTS	21
4.1 COMPOSITION - MVI-PEDIATRIC	65
5.1 ERYTHROCYTE TRANSKETOLASE ACTIVITY (TPPE %) INCUBATION CHART	72
5.2 ERYTHROCYTE TRANSKETOLASE ACTIVITY (TPPE %) PENTOSE UTILIZATION DETERMINATION	73
5.3 ERYTHROCYTE GLUTATHIONE REDUCTASE ACTIVITY ASSAY MEDIA	75
5.4 RADIOASSAY-FOLATE AND VITAMIN B ₁₂ PROCEDURE ...	79
5.5 URINARY THIAMIN ASSAY (THIOCHROME PROCEDURE) ..	81
6.1 DEMOGRAPHIC INFORMATION	88
6.1a PERCENT(%) TPN RECEIVED BY PARENTERAL GROUPS ..	91
6.2 VITAMIN CONCENTRATIONS IN TPN ADMIXTURE AND MVI-PEDIATRIC	95
6.3 DIETARY THIAMIN	98
6.4 ERYTHROCYTES TRANSKETOLASE ACTIVITY (TPPE %) ..	100
6.4a TRANSKETOLASE ACTIVITY	101
6.5 URINARY THIAMIN	103
6.6 THIAMIN BALANCE	104
6.7 DIETARY RIBOFLAVIN	107

6.8	EGR ACTIVITY (AC)	109
6.9	URINARY RIBOFLAVIN	111
6.10	RIBOFLAVIN BALANCE	112
6.11	DIETARY FOLATE	115
6.12	DIETARY FOLATE AT TIME 0 VS DIETARY FOLATE AT TIME 24 FOR THE PARENTERAL GROUPS	116
6.13	PLASMA FOLATE	118
6.14	RBC FOLATE	120
6.15	URINARY FOLATE	122
6.16	DIETARY VITAMIN B ₁₂	125
6.17	PLASMA VITAMIN B ₁₂	127

LIST OF FIGURES

FIGURE	PAGE
3.1 STRUCTURE OF THIAMIN HCL	24
3.2 STRUCTURE OF RIBOFLAVIN	30
3.3 STRUCTURES OF (1) RIBOFLAVIN 5-PHOSPHATE (FMN) AND (2) FLAVIN ADENINE DINUCLEOTIDE (FAD)	30
3.4 STRUCTURE OF (1) FOLIC ACID AND (2) 5-METHYL- TETRAHYDRO-FOLATE PENTAGLUTAMATE	40
3.5 STRUCTURE OF CYANOCOBALAMIN	55
5.1 METHOD OF STANDARD ADDITIONS	84
6.1 WEIGHT VS TIME	92
6.2 ENERGY INTAKE VS TIME	93
6.3 DIETARY THIAMIN VS TIME	97
6.4 RBC TPPE VS TIME	99
6.5 URINARY THIAMIN VS TIME	102
6.6 DIETARY RIBOFLAVIN VS TIME	106
6.7 EGR ACTIVITY VS TIME	108
6.8 URINARY RIBOFLAVIN VS TIME	110
6.9 DIETARY FOLATE VS TIME	114
6.10 PLASMA FOLATE VS TIME	117
6.11 RBC FOLATE VS TIME	119
6.12 METHOD OF STANDARD ADDITIONS URINARY FOLATE (INFANTS)	121
6.13 METHOD OF STANDARD ADDITIONS URINARY FOLATE (ADULTS)	121
6.14 DIETARY VITAMIN B ₁₂ VS TIME	124
6.15 PLASMA VITAMIN B ₁₂ VS TIME	126

CHAPTER 1.0 INTRODUCTION

Twenty years ago the survival rate of infants weighing less than 1500 g at birth was 20% (Tsang, 1988). Today 90 - 95% of these infants survive. Improved neonatal intensive care (e.g. assisted ventilation or continuous positive airway pressure, monitoring of heart rate and respiration, monitoring of blood gases and intravenous feeding) appears to be responsible for this marked increase in the survival rate (Halliday et al, 1984).

Optimal nutrition is critical in the management of the increasing number of surviving low birthweight (LBW) infants. Infants' organ growth and development (especially the brain) depends on adequate nutrition (Brooke, 1987). When the health of the infant is further compromised by complications such as surgery, respiratory distress syndrome, or the need for ventilatory assistance, early enteral feeding may not provide adequate energy and nutrition. However with the development of total and supplemental parenteral nutrition, the most vulnerable infant can receive sufficient nutrients.

Parenteral alimentation can be an effective means of providing energy and nutrition in the LBW infant who cannot tolerate enteral feeds. LBW infants given supplemental and total parenteral nutrition can achieve positive nitrogen balance and growth rates which parallel the intrauterine growth curve (Chessex and Zebich, 1985; Zlotkin et al, 1981; Heird and Winters, 1975). However, after more than a decade

of clinical experience in this field, there is still a lack of agreement on specific nutrient requirements for the intravenously fed LBW infant. While protein and other nutrient requirements such as copper and zinc of LBW infants receiving parenteral nutrition have been studied extensively (Zlotkin et al, 1981; Zlotkin and Buchanan, 1983; Reichman et al, 1982), there remains a scarcity of data on the vitamin requirements of LBW infants receiving parenteral nutrition.

In 1975 the Nutrition Advisory Group of the American Medical Association established guidelines for the use of intravenous vitamins for infants and children up to eleven years of age [Committee on Nutrition/Nutrition Advisory Group (CON/NAG), 1979]. These recommendations are based on oral recommended daily allowances. It has been suggested (Kerner, 1983; Hodges and Dempsey, 1986; Shenkin, 1986) that these recommendations be modified for parenteral use for the following reasons: 1) There may be nutrient-nutrient interactions when the vitamin supplement is added to the parenteral nutrition solution which may alter the relative effectiveness of a specific vitamin; 2) Some vitamins may adhere to the intravenous tubing and containers or may undergo oxidative destruction as observed in the case of vitamin A (Smith et al, 1988); 3) Parenteral vitamins are delivered intravenously rather than enterally which may impair their activation and/or storage and allow for their rapid excretion in urine; and, 4) Oral recommendations are designed for the

needs of healthy individuals and do not take into account special needs or underlying disease states which may alter nutrient requirements.

The Nutrition Advisory Group (CON/NAG, 1979) suggested that LBW infants receive 65% of the amount of vitamins recommended for full term infants. Until recently there were no published investigations studying the adequacy of the recommendations for the LBW infant receiving parenteral nutrition. One complete study and several preliminary reports have addressed this issue regarding water soluble vitamin requirements. Moore and collaborators (1986) investigated the adequacy of MVI-Pediatric (McGraw Laboratories), a multi-vitamin preparation formulated using the American Medical Association guidelines, for the LBW infant, term infant, and children up to eleven years of age who were receiving short or long term TPN. They observed that the LBW infant given MVI-Pediatric had high blood levels of ascorbate, vitamin B₁₂, folate, niacin and pantothenate. McCoy et al (1985, abstract) analyzed whole blood B₆ levels in human milk, formula, and parenterally fed infants. The patients receiving TPN had high levels of pyridoxal and pyridoxal phosphate. These investigators concluded that commonly used multivitamin solutions have excessive amounts of vitamin B₆. Manser and Brodsky (1985, abstract) found that the recommended levels of folate and B₁₂ (50 µg and 5 µg, respectively) were excessive for low birthweight infants. These studies implied that there

may be excessive intakes of these vitamins which could be dangerous to an already high risk group of infants. It has been suggested (Moore et al, 1986) that further investigative work should be done to determine more appropriate dosage levels for LBW infants receiving TPN.

Our hypothesis is that providing 40% of the amount of water-soluble vitamins recommended for full term infants will maintain normal biochemical functioning in LBW infants receiving total parenteral nutrition (TPN). Therefore, the purpose of this investigation was to determine the adequacy of 60% (the amount currently used at the Janeway Child Health Centre, St. John's, Newfoundland) or 40% of the levels of thiamin, riboflavin, folate, and vitamin B₁₂ that are recommended for full term infants for the parenterally fed LBW infant.

Chapter 2.0 LOW BIRTHWEIGHT (LBW) INFANTS

2.1 DEFINITION OF PREMATURITY

For a better understanding of the problems associated with investigating the vitamin requirements of the LBW infant, insight into the definition of prematurity and factors affecting the metabolism of vitamins is essential.

A preterm infant is any infant who is born prior to 37 weeks gestation. A LBW infant is born with a weight less than 2500 g whereas a very low birthweight infant is born weighing less than 1500 g (Halliday et al, 1985). Optimal nutrition is crucial in the management of LBW infants. Most of the serious nutritional problems are the result of immaturity, generally in those infants less than 30 weeks gestation. Physiological handicaps which may play a role in affecting nutrient needs and the metabolic handling of vitamins for the LBW infant are listed in Table 2.1.

2.2 PHYSIOLOGICAL HANDICAPS ASSOCIATED WITH PREMATURITY

LBW infants have difficulty maintaining body temperature due to an increased heat loss as a result of decreased subcutaneous fat and a large surface area to body weight ratio. As a result they are at risk for neonatal cold stress (Halliday et al, 1985). Cold stress is associated with acidosis and hypoxia (Stephenson et al, 1970; Gandy et al, 1964); two factors which impair surfactant production in LBW infants. In addition the very low birthweight infant has a

TABLE 2.1
PHYSIOLOGICAL HANDICAPS OF THE LBW INFANT

TEMPERATURE INSTABILITY
RESPIRATORY DIFFICULTIES
GASTROINTESTINAL AND NUTRITIONAL PROBLEMS
HEPATIC IMMATURITY
RENAL IMMATURITY
CEREBRAL IMMATURITY
IMMUNOLOGICAL DISTURBANCES
CARDIOVASCULAR DISORDERS

greater potential for fluid and electrolyte imbalance compared to larger LBW infants due to increased insensible water loss (Sarsohn, 1977). This water loss must be controlled to prevent dehydration and hypernatremia.

Fourteen to twenty per cent of babies born weighing less than 2500 g (Morley, 1986) develop respiratory distress syndrome (RDS) after birth. Twice as many males are affected as females. RDS results from a deficiency of lung surfactant. During the early management of RDS, infants should not be fed orally or nasogastrically for the following reasons; (i) Infants with RDS often are unable to absorb their feeds satisfactorily. This increases the likelihood of gastric distension leading to vomiting and aspiration of milk into the lungs; (ii) LBW babies have uncoordinated esophageal peristalsis and easily regurgitate milk from their stomachs (Morley, 1986); (iii) These infants also have poor laryngeal protective reflexes that result in a reduced ability to prevent tracheal aspiration of milk which may lead to a deterioration in their respiratory condition. Consequently, all infants suffering from RDS are given parenteral fluids until their respiratory distress subsides.

Poor sucking and swallowing reflexes, especially prior to 34 weeks gestation, result in oral feeding difficulties for the LBW infant. Therefore alternate modes of feeding have been developed to provide total nutrition for these infants.

Nutrient absorption is impaired, especially the digestion and absorption of fat, calcium, fat-soluble vitamins, and some

of the trace elements. Body levels of calcium, phosphorus, proteins, vitamins A, C, and E, trace elements and iron are less than in term infants and deficiencies of these nutrients may occur with growth (Halliday et al, 1985).

The pathways of intermediary metabolism of several amino acids in the liver of the LBW infant are incomplete or function inadequately. For example, the last enzyme in the transsulfuration pathway, cystathionase, is absent or reduced in the fetal liver and it appears only slowly after birth in LBW infants (Brooke, 1987). Therefore, the synthesis of cysteine is reduced causing this amino acid to be considered essential for this group. The absence of cystathionase also limits the tolerance to methionine which would normally be converted to cysteine if given in excess (Komrower and Robins, 1969). LBW infants fed high protein diets are more susceptible to hypermethioninemia which may result in neurological disturbances (Komrower and Robins, 1969).

Impaired conjugation and excretion of bilirubin results in hyperbilirubinemia which may lead to physiologic jaundice. Phototherapy is effective in reducing the serum unconjugated bilirubin concentration in newborns (Cremer et al, 1958; Lucey, 1972). One complication of phototherapy is the possible degradation of riboflavin (Sisson, 1987; Bates et al, 1985).

The immature brain is vulnerable, as well, to toxic effects of raised concentrations of certain amino acids such as phenylalanine, methionine, histidine, and the branched

chain amino acids (Brooke, 1987).

Due to renal immaturity, LBW infants have difficulties in excreting large solute loads and thus, a relatively dilute urine is produced. "Late metabolic acidosis" may develop from a disproportion between the daily load of nonvolatile acid generated in the body and the kidneys' ability to excrete it (Tuck, 1986). A low renal threshold for glucose results in persistent glycosuria in spite of normal blood sugar concentrations in some LBW infants. This limits glucose intake in intravenous feeding and may cause osmotic polyuria with disturbance of water balance (Brooke, 1987).

LBW infants have a reduced ability to combat infection due to the absence of placental transmission of immunoglobulins (IgM, IgA), relative inability to produce antibodies, impaired phagocytosis and decreased inflammatory response (Halliday et al, 1985). Infection frequently results in apnea, lethargy, gastrointestinal distention, vomiting, acidosis, and hypotension (Sarasohn, 1977).

The LBW infant is also at risk for persistent ductus arteriosus (PDA). The incidence of PDA in LBW infants is between 7 and 35% for all neonates weighing less than 1500 g (Levin et al, 1975; Rowe et al, 1981). The ductus arteriosus is a blood vessel which allows blood to bypass the lungs during intrauterine life. The physiological closure of this vessel usually occurs 24 hours after birth for a full term infant but may remain open in the preterm infant. The failure of the ductus to close normally may be due to a combination

of hypoxia (often as a result of RDS) and immaturity (Wilkinson and Cooke, 1986).

2.3 METHODS OF FEEDING

There is no general agreement about the ideal method to provide feedings for LBW infants. The method chosen depends upon the gestational age, birthweight, and clinical condition of the infant. The feeding of LBW infants is usually done in two phases; 1) the first phase is a period of instability and illness in which intragastric feeds are not tolerated and the provision of fluids and nutrients intravenously is necessary, and 2) the period of rapid growth during which enteral feeds are better tolerated.

2.3.1 PARENTERAL NUTRITION

Total parenteral nutrition (TPN) is used to maintain adequate nutrition and a positive nitrogen balance by the intravenous administration of most essential nutrients required by the body. LBW infants with severe respiratory disease, congenital anomalies of the gastrointestinal tract or inflammatory disease of the intestinal mucosa (i.e. necrotizing enterocolitis) are candidates for this mode of support.

The earliest reported use of intravenous feedings for neonates was during the 1930's (Pereira and Glassman, 1983). The peripheral intravenous infusion of protein (casein), protein hydrolysates, homogenized oils and lecithin emulsions,

and dextrose into severely malnourished infants for several days achieved positive nitrogen balance and weight gain. In 1966 Dudrick developed a more refined mixture of dextrose and amino acids that was administered through a central venous catheter that also resulted in normal growth and development in infants. Intravenous fat emulsions along with vitamin formulations were added to parenteral solutions in the 1970's and more recently trace minerals were included.

Positive nitrogen balance has been observed in LBW infants with parenteral lipid and/or glucose energy intakes of 60 kcal/kg/d and amino acid infusions of 2.5-3.0 g/kg/d (Anderson et al, 1979). It appears that growth requires a minimal parenteral nonprotein energy intake of 70 kcal/kg/d. Problems arise if glucose is used as the sole nonprotein energy source. At glucose concentrations greater than 13 g/dL there appears to be local irritation of peripheral veins resulting in tissue necrosis (Cowett et al, 1979). LBW infants also have poor glucose tolerance during their first few days of life with hyperglycemia appearing frequently at infusion rates greater than 6 mg/kg/min (Dweck and Cassady, 1974). Thus, it is recommended that glucose infusions begin at 6 mg/kg/min for infants weighing less than 1000 g and infusions not exceeding 8 mg/kg/min for those weighing between 1000 - 1500 g (Lifschitz, 1988).

There are three advantages in providing intravenous lipid preparations to LBW infants; 1) it is a source of essential fatty acids, 2) it has a high caloric density, and 3) its low

osmolality makes it suitable for peripheral vein use. However, LBW infants have a decreased ability to clear both free fatty acids and triglycerides from their serum. This impaired clearance of triglycerides may be secondary to a decreased activity of lipoprotein lipase resulting from reduced adipose tissue mass or hepatic immaturity (Pereira and Glassman, 1983).

The nitrogen in parenteral solutions is usually provided as a mixture of crystalline amino acids. It has been shown that the nitrogen of amino acid mixtures is utilized better than the nitrogen of protein hydrolysates (Duffy et al, 1981). Inadequate provision of amino acids is associated with failure to thrive, hypoalbuminemia and edema. Excessive amino acid intake can lead to hyperammonemia, metabolic acidosis, and possibly cholestatic jaundice (Yu, 1986; Pereira and Glassman, 1983).

Suggested daily intakes of minerals and vitamins are presented in Table 2.2. The justification for the use of TPN has been established in terms of energy and growth requirements for the LBW infant but little is known of the requirements and utilization of micronutrients.

The single most problematic gastrointestinal complication of TPN is liver disease which presents clinically as jaundice, and histologically as cholestasis (Pereira et al, 1981). Although one-third of LBW infants receiving TPN develop elevated liver function tests indicative of liver disease, the etiology remains obscure.

TABLE 2.2

**SUGGESTED DAILY INTAKES OF VITAMINS* AND TRACE MINERALS+
FOR LBW INFANTS RECEIVING TPN**

Nutrient	Recommended Amount unit dose/kg/d
Zn (μ g)	300
Cu (μ g)	200
Fe (μ g)	704**
Mn (μ g)	2-10
	unit dose/d
Vitamin A (IU)	1380
Vitamin D (IU)	240
Vitamin E (IU)	4.2
Vitamin K (μ g)	0.12
Thiamin (mg)	0.72
Riboflavin (mg)	0.84
Niacin (mg)	10.2
Panthothenic Acid (mg)	3
Pyridoxine (mg)	0.6
Vitamin C (mg)	48
Folate (μ g)	84
Vitamin B ₁₂ (μ g)	0.6
Biotin (μ g)	12

*These levels are recommended on the manufacturers' product insert of MVI-Pediatric in Canada. However, the American Medical Association revised their recommendation in 1988.

+CON/AMA, 1985.

**James et al., 1975.

2.3.2 GASTRIC ENTERAL FEEDING

Aggressive intravenous feedings should not preclude enteral feeding since even small amounts can stimulate gastrointestinal enzymatic development and activity, promote bile flow and increase small intestinal villous growth (Yu, 1987).

The method of providing bolus feeding into the stomach at frequent intervals (1 to 4 hours) has been commonly used in neonates for decades. Bolus feedings, however, may not be well tolerated by infants with delayed gastric emptying or respiratory problems. Pitcher-Wilmott and coworkers (1979) demonstrated a transient decrease in arterial oxygen tension and lung volumes in LBW infants recovering from respiratory distress who were receiving bolus feedings. Continuous gastric feedings may be tolerated better than intermittent gavage feedings especially if gastric emptying is not limiting in the infant (Landwith, 1972; Parker, 1981).

In 1970, Rhea and Kilby described a new technique in which feedings were delivered continuously by a tube passed either orally or nasally and advanced through the pylorus into the distal duodenum or jejunum. However, there have been criticisms of the transpyloric mode of feeding. The complications associated with transpyloric feeding include; 1) bacterial overgrowth of the upper intestine (Challacombe, 1974), 2) decreased absorption of potassium and fat (Roy et al, 1977), and 3) dumping syndrome and diarrhea (Heird, 1974). Controlled studies failed to demonstrate advantages

of using the transpyloric method of feeding over nasogastric techniques (Pereira and Lemons, 1981).

The American Academy of Pediatrics (1985) recommended intermittent gavage feedings as the method of choice for low birthweight infants but transpyloric and continuous gastric feedings could be considered for cases of extreme prematurity, RDS, delayed gastric emptying time and severe gastroesophageal reflux.

2.3.3 TYPES OF MILK AVAILABLE FOR ENTERAL FEEDING

2.3.3.1 HUMAN MILK

The nutritional adequacy of human milk for LBW infants is not as well established as it is for full term neonates. Pooled human milk was the preferred mode of feeding for LBW infants until the 1940's. Studies have shown, however, that pooled human milk from mothers of term infants does not meet all the nutritional requirements of LBW infants and results in a slower rate of growth than is found with the intake of their mother's own milk or commercial formulas. Fomon et al (1977) and Davies et al (1977) have suggested that banked human milk is not appropriate for LBW infants due to less than satisfactory concentrations of protein, minerals, and calories.

The milk of mothers of preterm infants (PTM) is qualitatively different from the milk of mothers of full term

babies (FTM). Since 1978 there has been a series of papers describing the differences in composition of PTM and FTM (Atkinson, 1978, 1980, 1981; Lemons, 1982; Gross, 1980) (Table 2.3). There are differing opinions regarding the relative concentrations of lactose, fat, calories and sodium. Atkinson et al (1978, 1980, 1981) reported that during the first month of lactation there was higher nitrogen, fat, and caloric content in PTM than FTM and lower concentrations of lactose. However, sodium, chloride, calcium, phosphate, potassium, and magnesium levels were similar in both milks. Lemons and coworkers (1982) found significantly greater protein, sodium, chloride, magnesium, and iron levels in PTM whereas lactose, fat and energy concentrations were similar. Protein and nonprotein nitrogen have consistently been found higher in PTM.

The water-soluble vitamin content of human milk is readily influenced by maternal diet. Ascorbic acid, nicotinic acid, riboflavin, thiamin, pantothenic acid, vitamins B₁₂ and B₆ are all relatively responsive to the recent maternal diet (Ford, 1983). In contrast, the fat-soluble vitamin content of milk appears to reflect both maternal vitamin stores arising from past diet intake as well as current diet (Anderson, 1985).

LBW infants fed PTM during the first month of life exhibit growth rates similar to those of infants fed whey protein formula and demonstrate positive retention rates of nitrogen, sodium, potassium, and chloride (Anderson, 1985; Pereira and

TABLE 2.3

COMPOSITION OF PTM AND FTM DURING THE FIRST MONTH OF LACTATION*

NUTRIENTS (units)	DAYS OF LACTATION					
	3-5		8-11		Mature	
	FTM	PTM	FTM	PTM	FTM	PTM
Energy (kcal/dL)	48±9	58±7	59±6	71±8	62±2	70±9
Protein (g/dL)	1.87±0.18	2.10±0.23	1.70±0.18	1.86±0.19	1.29±0.09	1.41±0.08
Lactose (g/dL)	5.14±0.70	5.04±0.43	5.98±0.73	5.55±0.35	6.51±0.56	5.97±0.35
Cu (mg/L)	0.72±0.13	0.83±0.21	0.73±0.21	0.78±0.18	0.58±0.09	0.63±0.14
Fe (mg/L)	1.11±0.43	1.10±0.34	0.99±0.31	0.99±0.27	0.88±0.28	0.90±0.23
Zn (mg/L)	5.35±1.20	5.30±1.45	4.10±0.65	4.75±1.56	2.60±0.65	3.92±1.10
Na (g/L)	0.43±0.09	0.53±0.20	0.32±0.12	0.46±0.14	0.25±0.09	0.28±0.10
Cl (g/L)	0.82±0.02	0.84±0.29	0.64±0.19	0.78±0.19	0.46±0.11	0.46±0.16
K (g/L)	0.72±0.11	0.72±0.09	0.63±0.09	0.68±0.11	0.57±0.09	0.57±0.07
Ca (g/L)	0.32±0.04	0.33±0.09	0.29±0.03	0.30±0.10	0.26±0.05	0.28±0.04
P (g/L)	0.12±0.05	0.14±0.05	0.15±0.03	0.15±0.04	0.13±0.01	0.15±0.04

TABLE 2.3 (CONT'D)

Mg (mg/L)	29.90±4.99	33.00±5.30	27.70±2.70	34.00±7.8	29.45±2.90	34.30±5.10
Carotene (µg/dL)	117±35	117±35	-	-	20±17	31±22
Retinol ester (µg/dL)	200±40	163±40	-	-	87±21	121±29
**Riboflavin (µg/dL)	28.8	27.6	27.9	36.0	31.0	26.6
**Thiamin (µg/dL)	2.84	2.37	6.46	5.40	18.30	8.93
**Vitamin B ₆ (µg/dL)	1.70	1.11	3.49	2.58	10.71	6.22
**Vitamin B ₁₂ (µg/dL)	0.05	0.09	0.03	0.05	0.02	0.02
**Folic acid (µg/dL)	1.76	1.06	3.12	2.09	4.23	3.05

*Adapted from Anderson (1985).

**Means but no standard deviation were available.

Barbosa, 1986). However, the concentrations of calcium, phosphorus, sodium, iron, copper, and zinc in PTM are still considered insufficient for this group (Mendelson et al, 1983; Pereira and Barbosa, 1986).

The anti-infection qualities of human milk are important attributes when considering desirable feeding choices for LBW infants. Gross (1981) observed that PTM contained higher amounts of IgA than FTM. Secretory IgA inhibits the adherence and proliferation of bacteria at epithelial surfaces and is important in controlling the microbial environment of the intestinal tract.

It is clear that PTM has advantages (i.e. greater nutrient density, immunologic properties and easier digestibility) over FTM as well as formula, at least during the first month of life of LBW infants.

2.3.3.2 COMMERCIALLY PREPARED FORMULA

As an alternative to human milk, proprietary formulas are complete foods requiring no supplementation. Results of feeding and growth studies led to the development of commercial formulas for LBW infants (American Academy of Pediatrics, 1977). The common features of these formulas include whey-predominant protein, carbohydrate mixtures of lactose and glucose polymers and fat mixtures containing combinations of medium chain triglycerides (MCT). A comparison of the nutritional content of these formulas is

shown in Table 2.4. The vitamin levels in the formulas are equal to or higher than those found in human milk. The stability of some of the vitamins is limited and a substantial reduction of biological activity may occur following heat treatment, canning, or light exposure (Orzalesi, 1987).

TABLE 2.4

NUTRIENT COMPOSITION OF PROPRIETARY INFANT FORMULAS FOR LOW BIRTHWEIGHT INFANTS

Component	Enfamil premature	SMA "preemie"	Similac Special Care
Protein (g/dL)	2.4	2.0	2.2
Whey casein ratio	60:40	60:40	60:40
Fat (g/dL)	4.1	4.4	4.4
MCT	40%	12%	50%
Oleo oil	0	20%	0
Corn oil	40%	0	30%
Oleic oil	0	25%	0
Coconut oil	20%	25%	20%
Soy oil	0	18%	0
Carbohydrate (g/dL)	8.9	8.6	6.6
Lactose	40%	50%	50%
Glucose polymers	60%	50%	50%
Minerals			
Calcium (mg/dL)	95	75	144
Phosphorus (mg/dL)	48	40	72
Sodium (mg/dL)	32	32	35
Potassium (mg/dL)	90	75	100
Chloride (mg/dL)	69	53	65
Magnesium (mg/dL)	8	7	10
Zinc (mg/dL)	0.8	0.5	1.2
Copper (mg/dL)	0.073	0.070	0.2
Manganese (mg/dL)	0.021	0.020	0.020
Iron (mg/dL)	0.13	0.3	0.3
Iodine (mg/dL)	0.006	0.008	0.015
Vitamins			
A (IU/L)	2540	3200	5500
D (IU/L)	507	510	1200
E (IU/L)	16	15	30
C (mg/L)	69	70	300
B ₁ (mg/L)	0.63	0.8	2
B ₂ (mg/L)	0.74	1.3	5
Niacin (mg/L)	10.1	6.3	40
B ₆ (mg/L)	0.53	0.5	2
B ₁₂ (μg/L)	2.5	2	4.5
Folic acid (μg/L)	240	100	300
K (mg/L)	0.08	0.07	0.1
Cal/dL	81	81	81

CHAPTER 3.0 VITAMIN METABOLISM IN HUMANS

3.1 INTRODUCTION

Vitamins are essential for the growing infant. Presently recommendations for the healthy full term infant are based on vitamin intakes from human milk (Committee on Nutrition, American Academy of Pediatrics, 1976). For the LBW infant a safety factor is added to these recommendations (Tsang, 1988). However, the assumption that the LBW infant needs will be met with adjustments for energy intake or body weight is no longer considered valid. LBW infants may be more vulnerable to develop vitamin deficiencies than full term infants because of predisposing factors such as lower body stores, decreased availability or intake, decreased absorption and an increased need or utilization (Orzalesi and Colarizi, 1982). Furthermore, the effects of excess vitamin administration for therapeutic reasons in LBW infants are unknown.

Whether nutrients are given by enteral or parenteral routes can also affect vitamin requirements. Guidelines for the intravenous use of vitamins are based on oral requirements (American Medical Association, 1979). However, parenteral requirements for vitamins may be quantitatively different from oral requirements for reasons already mentioned (Chapter 1.0). Moore et al (1986) addressed the appropriateness of the present recommended intravenous intake of certain water soluble vitamins. They determined serum levels of water

soluble vitamins and certain functional indices of vitamin status in infants and children, including 18 LBW infants, receiving an intravenous multivitamin preparation (MVI-Pediatric). While there was no matched enterally fed group, nor premature cord blood samples, the serum levels of most water soluble vitamins in LBW infants did exceed several-fold that of term infants and older children. Assays to determine the adequacy of thiamin, riboflavin, and pyridoxine, were conducted and it was found that there was no apparent deficiency at the recommended levels of intake. In a preliminary study, Manser and Brodsky (abstract, 1985) indicated that vitamin B₁₂ and folate at intravenous intakes of 5 µg/d and 50 µg/d, respectively, resulted in apparent excessive levels of both vitamins. Greene and coworkers (abstract, 1988) evaluated the plasma and red cell riboflavin levels as well as flavin adenine dinucleotide (FAD) concentrations in infants less than 1.5 kg who received TPN for the first 3-4 weeks of life. Their study indicated that plasma riboflavin levels increased 40-100 fold with the currently recommended dose of riboflavin and stated that a reduced renal clearance as well as an excessive intake were important determinants of this phenomenon.

3.2 THIAMIN METABOLISM

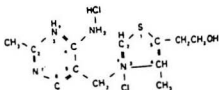
Thiamin (Figure 3.1) is important as a constituent in many of coenzymes of carbohydrate and amino acid metabolism.

The most abundant form of thiamin in animal tissues is thiamin pyrophosphate (TPP), a coenzyme which plays an integral part of two general enzymatic sequences; 1) decarboxylation of α -ketoacids and; 2) transketolation in the pentose phosphate pathway yielding NADPH and ribose from the metabolism of glucose (Marks, 1985).

In the Kreb's cycle two thiamin dependent reactions occur. Pyruvic acid is converted to acetyl CoA through the action of

Figure 3.1

Structure of Thiamin HCl



pyruvate dehydrogenase in association with coenzyme A and lipoic acid. The conversion of α -ketoglutarate to "active succinyl semi-aldehyde" is catalyzed by α -ketoglutarate dehydrogenase which, through the participation with lipoic acid and coenzyme A, yields succinyl coenzyme A.

Transketolase catalyzes two reactions in the pentose phosphate pathway. The ketol groups of appropriate keto-sugars form an active glycoaldehyde-enzyme intermediate with TPP which is then transferred to a suitable acceptor aldehyde. Consequently, transketolase is capable of

functioning in a number of donor-acceptor systems. However, the main action of this enzyme is related to ribulose-5-phosphate metabolism yielding ribose for nucleotide formation (Mock, 1986; Sauberlich, 1967).

Thiamin is rapidly and actively absorbed by the small intestine and is then, intracellularly phosphorylated to the active coenzyme, TPP. Rindi and coworkers (1972) observed that the human jejunal mucosa absorbs thiamin at low concentrations by an active, carrier-mediated process that can accumulate thiamin against a concentration gradient. In fact, two transport systems have been demonstrated in the intestinal mucosa; 1) passive diffusion at thiamin concentrations greater than 2 μM , and 2) an active saturable sodium-dependent process at concentrations less than 2 μM (Komai et al, 1974). Factors such as anoxia, sodium deficiency, low temperatures and excessive alcohol consumption decrease thiamin absorption (Gluber, 1984). Thiamin preparations with greater lipid solubility (i.e. thiamin alkyl disulphides) are much more readily absorbed and thus, result in higher blood and tissue levels than found with preparations containing thiamin hydrochloride (Gluber, 1984).

The body is incapable of storing free thiamin but small amounts of the phosphorylated form are present in all cells. In fact, most nonlipid tissues contain approximately 1 μg thiamin/g tissue (Greene, 1981). Whole blood has been reported to contain 5-8 $\mu\text{g}/100\text{ mL}$ in adults. Levels less than

4 $\mu\text{g}/100 \text{ mL}$ are an indication of a thiamin deficiency. Henshaw and associates (1970) found erythrocytes to contain $22.5 \pm 5.88 \mu\text{g}/100 \text{ mL}$ in a group of college women. However, blood thiamin may not be useful in detecting thiamin deficiency for the following reasons; (i) there is usually only a small reduction in blood thiamin during deficiency states, (ii) there are technical difficulties associated with its measurement, and (iii) whole blood thiamin concentrations are a reflection of the immediately preceding intake and may not be a reliable index of tissue stores, distribution, or actual biochemical functioning. Thus, the level of blood thiamin may be of limited value for the interpretation of actual thiamin status (Sauberlich et al, 1974).

Excess dietary thiamin leads to an increase in urinary thiamin output. The determination of daily urinary thiamin excretion has been used to estimate thiamin status but these values only reflect recent intake and not body stores. Urinary thiamin decreases proportionately to thiamin intake to a threshold point after which further lowering of intake results in only minor and variable changes in urinary excretion. Intakes of thiamin less than the threshold critical point will cause depletion of tissue stores of the vitamin and could result in symptoms of deficiency (Sauberlich et al, 1974). If an adult consumes $0.5 \text{ mg}/1000 \text{ kcal/d}$, $100 \mu\text{g}$ of thiamin is excreted in the urine. If the intake is lowered to $0.36 \text{ mg}/1000 \text{ kcal/d}$, then the output is reduced to

40-50 μg (Saublerlich et al, 1974). Knowledge concerning urinary excretion levels in children is limited but output levels of 40-100 $\mu\text{g}/\text{d}$ or 176 $\mu\text{g}/\text{g}$ creatinine have been reported (Greene, 1981). Stearns et al (1958) concluded that children consuming 0.5 mg of thiamin or less excreted about 10% of their daily intake. At intake levels above this, the mean output value was approximately 20%. A deficiency likely exists in children if urinary thiamin output is less than 15 $\mu\text{g}/24\text{ h}$ or less than 120 $\mu\text{g}/\text{g}$ creatinine (Greene, 1981).

More than twenty thiamin metabolites have been found in human urine. Of these, eight appear to be major (Saublerlich, 1967). None have been proven useful thus far for evaluating thiamin status. However, additional studies may reveal that the amount of thiamin metabolites excreted may represent a measure of the rate at which body stores of thiamin are depleted.

A dietary lack or inability to assimilate thiamin can result in the deficiency disorder, beriberi. Beriberi has a spectrum of manifestations ranging from severe cardiac failure in 'wet beriberi' to a variety of neurologic deficits in 'dry beriberi'. The commonly observed symptoms include general malaise, headache, nausea, generalized aching of muscles, vomiting, fatigue, anorexia and psychic and emotional disturbances such as irritability, moodiness, depression (Saublerlich, 1967). Neurologic symptoms of beriberi may not occur for several months after tissue levels of thiamin are

deficient because of the slow degeneration of affected nervous tissue. Reactions involving decarboxylation of α -ketôacids are more affected and accumulation of pyruvate, lactate, α -ketoglutarate, and glutamate occur in the serum and urine before neurologic symptoms develop (Greene, 1981). A condition called infantile beriberi has been observed in breast-fed infants predominantly in rice-consuming countries. These infants present with generalized edema, dyspnea, anorexia, vomiting, cardiac disturbances, oliguria, and aphonia or 'beriberi cry' (Sauberlich, 1967).

It is important to note that the metabolism and function of thiamin may be modified by changes in the levels of other nutrients. For instance, a rise in carbohydrate intake increases the requirement for thiamin whereas the requirement decreases when fat consumption is increased. Disturbances in thiamin function and a reduction in thiamin levels in tissues results from a decrease in magnesium, pyridoxine, and vitamin B₁₂. Folate deficiency leads to thiamin malabsorption. Thiamin deficiency can also be caused by chronic alcoholism (Clark et al, 1984).

In humans, no toxic effects of thiamin have been reported. However, anaphylaxis secondary to parenteral use has been observed but thiamin intakes of 100 mg or less are rarely associated with anaphylactic reaction (Aldaheff et al, 1984).

Based on nutrition surveys and experimental deficiencies, the adult requirement for thiamin has been estimated to be

0.2-0.23 mg/1000 kcal (Gluber, 1984). With the addition of a safety factor, 0.4 mg/1000 kcal appears to be adequate for most healthy adults (Health and Welfare Canada, 1983).

For infants there is little data available on thiamin needs even though thiamin is involved in neurological function, an area of great concern in the developing child. The suggested recommended oral intake for infants is 0.4 mg/1000 kcal (Health and Welfare Canada, 1983). This is higher than the level secreted in breast milk by well nourished mothers. Since the thiamin intake of breastfed infants appears satisfactory (0.3 mg/1000 kcal), the amount recommended should supply the basic need and an added safety factor.

3.3 RIBOFLAVIN METABOLISM

Riboflavin (Figure 3.2) is a yellow-green, fluorescent, water soluble pigment widely distributed in plant and animal cells (Shah, 1985; Marks, 1985; Mock, 1986). This vitamin is incorporated into two active coenzymes, flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) (Figure 3.3) which form the prosthetic groups of many different enzyme systems (flavoproteins) involved in the oxidative degradation of pyruvate, fatty acids and amino acids, electron transport and in drug metabolism. Flavoproteins are relatively unstable especially when tissue protein is depleted by physiologic stress, dietary deficiency, or disease. Under these

conditions increased levels of riboflavin are excreted in the urine (Greene, 1981).

Figure 3.2

Structure of Riboflavin

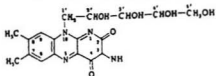
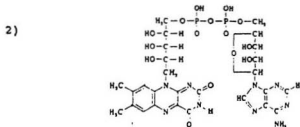
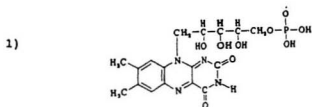


Figure 3.3

Structure of (1) riboflavin 5-phosphate (FMN) and (2) flavin adenine dinucleotide (FAD)



Riboflavin is absorbed in the small intestine by a site-specific, saturable, specialized transport process. The absorption of free riboflavin and FMN have been studied in both humans and rats (Marks, 1985; Greene, 1981). FMN is hydrolyzed in the upper gastrointestinal tract to free riboflavin which then enters the mucosal cells of the small intestine. Riboflavin is then phosphorylated to FMN which enters the portal system where it is bound to albumin, transported to the liver, and converted to FAD.

In human plasma, about one half of free riboflavin and 80% of FMN is bound to protein, primarily albumin. Spector (1980) observed that certain drugs (i.e. ouabain, theophylline, and penicillin) may displace riboflavin from the binding protein and therefore inhibit transport to the central nervous system.

Riboflavin is present in all cells only as functioning compounds and not as stored material, although the liver (with 16 $\mu\text{g/g}$ tissue) and kidney (with 25 $\mu\text{g/g}$ tissue) usually contain more than the other tissues which average about 2.3 $\mu\text{g/g}$ (Greene, 1981). Burch et al (1948) found the erythrocyte riboflavin content to be 20.2-27.6 $\mu\text{g}/100$ mL cells in supplemented subjects and 10.0-13.1 $\mu\text{g}/100$ mL in deficient subjects. In the various tissues, riboflavin is usually present as a phosphate or one of the coenzymes. In the retina, however, riboflavin is present in the free form, the significance being unknown (Marks, 1985).

Small quantities of this vitamin are lost in sweat and

excess amounts are eliminated in the urine. Studies of the turnover rate of riboflavin in the normal state have shown that the half life is about 16 days. Riboflavin has been thought to be excreted unchanged since no decomposition product has been found in the tissues or urine (Cooperman and Lopez, 1984).

Since the body metabolizes little riboflavin urinary excretion appears to correlate well with reserves and intake of the vitamin. When body stores in adults are saturated a daily intake of 1.3 mg results in almost quantitative excretion in urine. When body stores are depleted or when the daily intake is less than 1 mg, urinary excretion is reduced (Selhub and Rosenberg, 1984). Urinary output changes with age and pregnancy. In conditions of negative nitrogen balance, fasting, enforced bed rest, and heat stress, increased excretion levels have been observed whereas sleep and short periods of heavy physical work decrease riboflavin excretion (Sauberlich et al, 1974).

Isolated riboflavin deficiency is rare. It is common in association with pellegra, beriberi, and kwashiorkor (Selhub and Rosenberg, 1984). Clinical features of deficiency are photophobia, a burning ocular sensation, dim vision, and sore mouth and tongue. Nonspecific symptoms of anorexia, weight loss, weakness, headache, dizziness, and confusion may precede overt symptoms of stomatitis, pharyngitis, glossitis, seborrheic dermatitis, normochromic normocytic anemia and

corneal vascularization. In infants and children, dietary deficiency, which is most common in the spring and summer months, may lead to symptoms within one to two months (Greene, 1981).

Riboflavin deficiency may occur from a decrease in intake, malabsorption, or an increase in need especially during pregnancy, periods of rapid growth, acute or chronic infection, and trauma. Absorption of riboflavin is reduced in hepatitis, cirrhosis, and probenecid treatment (Greene, 1981).

Riboflavin toxicity has not been reported in animals or man. There may be interactions with drugs or other vitamins (Greene, 1981; Mock, 1986; Aldaheff et al, 1984). For instance, riboflavin may inhibit the uptake of methotrexate by neoplastic cells.

The minimum recommended intake for adults to maintain normal riboflavin output is 0.5 mg/1000 kcal (Health and Welfare Canada, 1983). Very little information is available on the riboflavin requirements of infants and children. Health and Welfare Canada (1983) recommend that infants should receive an oral intake of 0.5 mg/1000 kcal. Factors that influence riboflavin status and hence requirements include drugs, disease (i.e. thyroid and congenital heart disease), phototherapy, age, and other nutrients.

Ingestion of boric acid, probenecid, and oral contraceptive drugs may induce riboflavin deficiency. Boric

acid displaces riboflavin from plasma binding sites and results in increased urinary excretion of the vitamin (Roe et al, 1972) whereas probenecid inhibits gastrointestinal absorption and renal tubular secretion of riboflavin. Anticholinergic drugs delay gastric emptying and decrease intestinal transit rate allowing for an increase in riboflavin absorption (Jusko and Levy, 1975).

Thyroid disease affects riboflavin metabolism. Thyroxine stimulates intestinal motility and as a result riboflavin absorption is decreased in hyperthyroidism and increased in hypothyroidism (Rivlin, 1970a). Rivlin (1970b) has also reported that flavokinase activity is suppressed in hypothyroidism and liver levels of FAD and FMN are depressed. Since thyroid function in these infants could be immature then the possibility of an increased requirement must be considered. A high prevalence of riboflavin deficiency has been observed in children due to congenital heart disease (Steier et al, 1976). This condition is associated with deficiencies of other vitamins as well.

Treatment modalities for special conditions can affect vitamin requirements for the full term and LBW infant. Phototherapy, the treatment for neonatal hyperbilirubinemia, may also result in the photodegradation of riboflavin (Sisson, 1987; Bates et al, 1985). Riboflavin is especially sensitive to blue light (the light used for phototherapy) since its excitation maximum is at 450 nm. Thus the combination of low

body stores, low intakes, and phototherapy may result in a deficiency of riboflavin at a critical period for the newborn. Bates and coworkers (1985) examined the susceptibility of riboflavin and vitamin A in breast milk to photodegradation by exposing the milk to daylight and to phototherapy illumination under conditions similar to those encountered in the neonatal unit. They found up to 50% of riboflavin was destroyed when human breast milk was exposed to daylight or phototherapy either in translucent polythene bottles or where milk was pumped through nasogastric tubing from a syringe to mimic the conditions of enteral feeding. Smith et al (1988) investigated the effect of phototherapy light on the vitamin stability in TPN admixtures and found that there were significant decreases in the amount of riboflavin remaining after 48 hours. These losses were about the same for all the amino acid solutions used and in both plastic bags and glass flasks. The light-induced destruction of riboflavin tended to not be as great when Intralipid was included in the admixtures.

Is it the riboflavin in the body tissues which is destroyed by phototherapy or does light destroy the vitamin in the TPN solution? Clearly, it has been shown that light degrades the vitamin in the TPN admixture (Bates et al, 1985; Smith et al, 1988). However, in studies done on rats exposed to the sun for 20 hours during a course of 13 days, a significant decrease in total flavin concentration was

observed in blood (25% decrease), kidneys (40% decrease) and liver (60% decrease) while urinary excretion was increased (Maslenikova, 1963).

The use of phototherapy has been reported (Hovi et al, 1979) to produce a biochemical riboflavin deficiency in breast fed newborn infants as measured by the activation of erythrocyte glutathione reductase. Hovi et al (1979) studied 65 newborns; 28 healthy newborns and 37 newborns with hyperbilirubinemia prior to and during phototherapy. Their data indicated that healthy newborns given human milk which is relatively poor in riboflavin, had evidence of transient riboflavin depletion after birth. This effect was made more pronounced by phototherapy and was partially prevented by parenteral or oral administration of moderate amounts of riboflavin. Meloni and coworkers (1982), on the other hand, observed that phototherapy did not influence riboflavin status in normal or glucose-6-phosphate dehydrogenase deficient infants suffering from neonatal jaundice. The discrepancies in the studies could be due to the fact that infants observed by Meloni were exposed to phototherapy for less than 48 hours as opposed to the average of 3 days as in other investigations and these infants were formula fed as opposed to breast fed. Other factors to consider are the variability of maternal riboflavin intake and thus fetal stores at birth, differences in postnatal sampling ages, slight differences in laboratory methods for analyzing erythrocyte glutathione reductase

activation, feeding practises and vitamin supplementation.

It was recently shown (Lucas and Bates, 1984) that biochemical riboflavin deficiency is common in LBW infants. Lucas and Bates (1984) investigated the riboflavin status of 68 LBW infants receiving banked human milk (0.3 mg riboflavin/L) or being fed formula containing 1.8 mg riboflavin/L. Riboflavin status was determined by the erythrocyte glutathione reductase activity using an activity coefficient (AC) equal to 1.3 as the upper limit of normality. They concluded that the two factors which were associated with the biochemical evidence of impaired riboflavin status were the relatively late introduction of the multivitamin supplement and the use of human milk instead of riboflavin supplemented infant formula as the primary diet. These results suggest that the adequacy of riboflavin in the tissues of the premature newborn is borderline and may become insufficient if supplementation to the diet is not provided.

Is there a need, then, for riboflavin supplementation for LBW infants, especially those being fed human milk? Ronnholm (1986) analyzed the riboflavin status of 39 LBW infants. Nineteen received supplemented human milk in which 0.3 mg riboflavin was added daily to mother's or banked milk samples and 20 infants received no supplementation for a period of up to 12 weeks after delivery. At 6 weeks post delivery, 47% of those without supplementation had AC values indicative of a deficiency. The riboflavin status of infants being

supplemented was significantly better. Consequently, Ronnholm recommended that supplementation of infants most likely to develop riboflavin deficiency was useful but warned that dosage, time, and mode of administration to infants undergoing phototherapy must be carefully adjusted to avoid unwanted side effects. Further to this warning, it is interesting to note that riboflavin can act as a photosensitizing agent which can accelerate bilirubin photooxidation (Meizel et al, 1978). Sisson (1987) stated that intramolecular photoreduction of riboflavin occurs in the presence of light by donation of electrons from such compounds as amino acids, amines, and NADH. In the process of aerobic photoreduction, flavins catalyze the aerobic photooxidation of a number of substances by reversible photoreduction and photosensitization, in which latter action triplet flavin transfers energy to oxygen to yield singlet oxygen.

Because of these photochemical actions and the fact that flavins are present in nearly all biological fluids and tissues, riboflavin has a significant influence in neonatal phototherapy (Sisson, 1987).

However, studies by Ennever et al (1983a, 1983b) showed that standard phototherapy illumination in the presence of riboflavin produced single- and double- strand breaks in both purified and intracellular DNA by both oxygen dependent and independent reactions. Since light activated riboflavin has the propensity for tissue damage, it has been suggested that

caution be exercised in providing large supplements of this vitamin to neonates receiving phototherapy. Ennever and fellow workers (1983b) suggested that there is potential for the production of genetic damage even with the use of multivitamin solutions exposed to phototherapy. This is important since many LBW infants are fed intravenously with solutions containing multivitamin formulations at the same time they receive phototherapy.

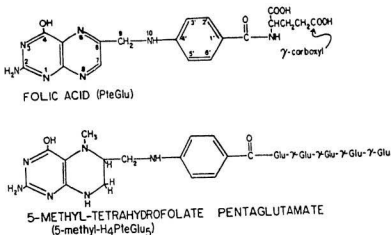
3.4 FOLATE METABOLISM

Folates (Figure 3.4) are found in a wide variety of plant and animal tissues mainly in the reduced methyl or formyl glutamate forms. Folic acid is reduced by a two step reaction to its coenzyme form (tetrahydrofolic acid) which serves as an intermediate carrier of hydroxymethyl, formyl, and methyl groups (Keagy, 1985; Rodriguez, 1980; Mock, 1986). Tetrahydrofolates serve as acceptors or donors of one carbon units which are involved in the following metabolic systems;

- 1) formation of glycineamide ribonucleotide and 5-amino-4-imidazole carboxamine ribonucleotide in early purine synthesis;
- 2) methylation of deoxythymidylic acid to thymidylic acid in pyrimidine nucleotide biosynthesis;
- 3) amino acid conversions [i.e. serine to glycine, histidine to glutamic acid, and homocysteine to methionine]; and
- 4) generation of formate and its utilization (Shinton, 1972).

Figure 3.4

Structure of folic acid and a 5-methyltetrahydrofolate pentaglutamate



Dietary folates are predominantly pteroylpolyglutamate (PteGlu) derivatives. The process of folate absorption requires their hydrolysis to monoglutamate derivatives prior to folate transport across the intestinal mucosa (Halsted, 1979). Pte-Glu is absorbed predominantly in the duodenum and jejunum by an active process stimulated by glucose and exhibiting saturation kinetics. At higher folate concentrations, absorption follows first order kinetics, suggesting the presence of a second passive component for transport (Brady et al, 1984). Metabolism of Pte-Glu to 5-methyl tetrahydroPteGlu during passage across the mucosa has been observed and has led to the postulation that mucosal metabolism plays a role in folate absorption. However, when

large doses of Pte-Glu are administered only a small proportion is methylated and much of the Pte-Glu can be recovered unchanged in the urine. Thus methylation does not appear to be a prerequisite for absorption (Brady et al, 1984).

Polyglutamates, the main intracellular form of folate, do not cross mammalian cell membranes. It has been suggested that polyglutamates enter the villous epithelial cells where the peptide chain is removed by α -carboxylpeptidases and then further reduced to tetrahydrofolates by folate reductases. In this form folates enter the portal circulation (Shinton, 1972). These monoglutamate derivatives are taken up by cells by specific transport systems and are metabolized intracellularly to polyglutamate forms which are retained by the cells.

The liver may be responsible for the production or maintenance of the major form of plasma folate, 5-methyl tetrahydrofolate. When humans are given oral Pte-Glu, the portal plasma initially contains mainly the nonmethylated folates, whereas peripheral plasma contains the methyl form at all times. Over time portal plasma folate assumes the methyl form as well. Thus, folate absorbed through the intestine enters the liver where it is converted to the methyl form and then most is released back into the circulation (Dencher et al, 1976).

Plasma levels vary from 3-21 ng/mL in healthy individuals

(Shinton, 1972). These values are sex and age independent but are affected by diurnal variation associated with meals. When dietary intake is reduced, plasma folate levels fall within a few days due to the cessation of the exchange mechanism observed with liver folate. Hoffbrand et al (1966) observed that patients with serum folate levels less than 3.0 ng/mL almost always had megaloblastic anemia or obvious red cell morphological changes of folate deficiency.

Total content of body folate in adults is 70 mg with one third (5-15 $\mu\text{g/g}$) contained in the liver. Liver folate falls to 1.5 $\mu\text{g/g}$ in about 130 days on a folate deficient diet with megaloblastic changes. Folate is incorporated in red blood cells during erythropoiesis and there is only a slight fall during their life span. Red blood cell folate is a useful indicator of body folate status with an average level of 300 ng/mL (range 160-640 ng/mL) (Shinton, 1972).

Folate is lost from the body in urine, feces, sweat and saliva. The daily urinary excretion of intact folates for adults is between 1-12 μg (Brady et al, 1984); this is approximately 1% of the daily dietary intake (Cooperman et al, 1970). The amounts excreted are increased after injected or oral test doses of folate. Measurements of urinary levels of folate, with or without folic acid test doses, have not been of value for evaluating folate nutritional status (Cooperman et al, 1970). An increase of formiminoglutamic acid (FIGLU) excreted in the urine after a histidine load, suggests a

folate deficiency. However, this test is not specific for folate since the same is observed with a vitamin B₁₂ deficiency (Herbert, 1983). Tracer studies in humans indicate an initial excretion of unmetabolized Pte-Glu together with 5-methyl-tetrahydroPte-Glu and 10-formyl-tetrahydroPte-Glu following oral or intravenous doses of labelled Pte-Glu (Brady et al, 1984). With time, the urine contains labelled breakdown products, presumably the acetamidobenzoylglutamate derivative and labelled pteridines. However, over one half of the radioactivity is excreted in the feces.

Denko et al (1946) reported that feces contain 5-15 times the amount of folate ingested. Possible sources of fecal folate include unab. bed dietary folate, folate resynthesized by oral and intestinal microflora, endogenous metabolism such as that which is excreted in bile and saliva or from degradation of gastrointestinal cells. Bile contains high levels of folate due to enterohepatic circulation of folate. Folate concentration in bile ranges from 10-89 ng/mL, which is two to ten times as much folate found in serum (Rodriguez, 1980). In humans, total body folate stores turnover at an estimated rate of less than 1% per day (Krumdieck et al, 1978). Most of the folate in bile is reabsorbed in the intestine. An estimated 0.1 mg folate is lost per day due to incomplete absorption of biliary folates in normal subjects (Brady et al, 1984). In cases of folate malabsorption, biliary folate loss may contribute significantly to ensuing

folate deficiency.

Folate deficiency may occur via one or more of the six possible etiologic mechanisms common to all nutritional deficiencies (i.e. inadequate ingestion, absorption, or utilization and/or increased requirement, excretion, or destruction) (Herbert, 1983).

Megaloblastosis, resulting from a deficiency of both folate and vitamin B₁₂, is due to a disturbance in DNA synthesis. Deoxyuridylate is inadequately converted to thymidylate because the amount of the coenzyme, 5,10-methylene tetrahydrofolate is reduced. In folate deprivation this reduction occurs because of the reduction of folate in the body whereas in vitamin B₁₂ deficiency there is a decreased amount of coenzyme (Herbert, 1983). Hypersegmentation of polymorph nuclei occurs after seven weeks of folate deprivation and during the eighteenth week macrocytic anemia is observed. Florid megaloblastic anemia with gross changes in bone marrow is associated with a shortened life span of cells produced and leads to hemolytic anemia with jaundice, leucopenia, and thrombocytopenia. Gastrointestinal disturbances include glossitis, stomatitis, and intestinal malabsorption. Other organs affected include the cervical and vaginal squamous epithelium, the ovaries and the testes, resulting in infertility (Shinton, 1972; Herbert, 1983).

Skin disorders (i.e. psoriasis, dermatitis, herpetiformis, rosacea, eczema, exfoliative dermatitis), obstetric disorders

(i.e. toxemia, accidental hemorrhage, abortion, and congenital anomalies), neuropathy, and psychiatric disorders are symptoms associated with folate deficiency (Marks, 1985).

The most common cause of megaloblastic anemia in infants and children is folate deficiency. Body folate stores at birth are small and are rapidly depleted by requirements for growth. Serum and red blood cell folate fall below adult levels by the second week of life and remain below the adult values for the first year of life (Herbert, 1983).

Little is known about the subclinical effects of excessive folic acid intake. Some researchers found that megadoses produced mental changes, sleep disturbances, gastrointestinal symptoms, malaise, and irritability. Others have not been able to confirm these observations (Rodriguez, 1980; Aldaheff et al, 1984).

If a vitamin B₁₂ deficiency exists, megadoses of folate will mask the anemia but do not prevent the irreversible damage to the nervous system which occurs (Aldaheff et al, 1984). Ford et al (1975) suggested that excess folic acid may have an adverse affect on the microbial population in the intestine of infants by increasing the growth of folate-requiring bacteria. High levels of folate may also interfere with the action of drugs. For instance, folate and the anticonvulsant, phenytoin, inhibit the uptake of each other at the gut cell wall and possibly at the blood brain barrier. Megadoses of folate may precipitate convulsions in

persons whose epilepsy is controlled by phenytoin (Herbert, 1987). It has also been observed that very large doses of folate given parenterally to experimental animals may precipitate in the kidneys producing kidney damage and hypertrophy (Herbert, 1987a).

The minimal daily requirement for adults is about 50 μg based on findings that daily parenteral administration of this amount corrects folate deficient anemia (Herbert, 1987a). It has been observed that about 90% of the North American population consume adequate folate for daily metabolic needs but also enough folate to sustain a substantial folate storage (more than 140 ng folate/mL red cells) against periods of dietary deprivation (Herbert, 1987a). From these observations the recommended daily intakes (RDI) for adults has been set at 3 $\mu\text{g/kg/d}$ (Health and Welfare Canada, 1983). Factors which affect folate requirements include growth, other dietary factors, certain drugs, and infection. Increases in the number of body cells or the rate of cell turnover affects folate requirements since folates are required for DNA and RNA synthesis. Therefore periods of growth, pregnancy, lactation and innumerable pathological conditions increase the requirement for folate (Rodriquez, 1980).

The availability of dietary folate may also affect the requirements. For instance, the chemical structure of folate, the cellulose content of the diet, naturally occurring antagonists, glucose and the pH may affect folate absorption

(Benn et al, 1971; Corcino, 1975; Gerson et al, 1971). There is some evidence that compounds such as iron, oral contraceptives, ascorbic acid, cobalamin, methionine, alcohol, and certain drugs plus vitamin B₁₂ deficiency interfere with the utilization and storage of folate (Rodriquez, 1980).

Many drugs affect folate metabolism and in some cases induce megaloblastic anemia. For instance, methotrexate, aminopterin, antimalarial drugs (pyrimethamine) and antibacterial drugs (trimethoprim) are inhibitors of dihydrofolate reductase (Rodriquez, 1980). Anticonvulsant drugs (diphenylhydantoin, primidane) and barbituates antagonize folate status (Rodriquez, 1980). Prolonged treatment with the bile sequestering agent, polidexide, will produce folate deficiency in children (Ritland et al, 1975).

The effect of infection on folate nutriture is not well established. Folate deficiency in people with infections has been attributed to low reserves, inadequate intake, loss through emesis and diarrhea, increased granulocyte and red cell turnover and impaired absorption or utilization (Luhby, 1959; Mac Iver and Back, 1960; Zuelzer and Ogden, 1946). Many clinicians (Kende et al, 1963; Khalil et al, 1973; Mac Iver and Back, 1960; Zuelzer and Rutzky, 1953) have reported megaloblastic anemia due to folate deficiency in infants and children with diarrhea, gastroenteritis, and respiratory infections, especially in LBW infants and infants less than six months. The diarrhea ceased when folate was administered.

Others have reported that diarrhea and infections preceded the development of megaloblastic anemia suggesting that increase folate requirements due to infection precipitated the anemia. Luhby (1959) concluded that in infants 2-5 months old severe infection was the principle cause of megaloblastic anemia whereas in infants 8-12 months, inadequate diet was the principle factor.

Infants require about ten times as much folate as adults on the basis of body weight. The recommended intake of 30-65 $\mu\text{g}/\text{d}$ (Dallman, 1988) is provided by breast milk which supplies approximately 50 $\mu\text{g}/\text{L}$ (Herbert, 1987a; Dallman, 1988). The RNI for Canadians recommends an oral intake of 50 $\mu\text{g}/\text{d}$ for infants (Health and Welfare Canada, 1983). Even though there is little storage of folate at birth (Herbert, 1988), infants fed human milk or proprietary cow milk formulas are at little risk for developing a folic acid deficiency (Ek and Magnus, 1980; Ek and Magnus, 1982; Salmenpera et al, 1986; Tamura et al, 1980).

Salmenpera and coworkers (1986) conducted an investigation to assess whether prolonged exclusive breastfeeding provided adequate folate nutrition for full term infants. Two hundred Finnish mothers and their offspring participated in this study. Infants who were weaned from breast milk received formula containing 35 μg folate/L and folate status was determined by analyzing the plasma folate concentration. The authors found that the exclusively breast fed infants had

adequate plasma folate concentrations whereas after being weaned plasma folate levels dropped rapidly in the formula fed group and rose only after solid food was introduced. Salmenpera et al (1986) concluded that the breast fed babies were protected against folate deficiency whereas formula fed infants may have marginal intakes even if the formula contains the recommended level of folate. However, there are three serious problems concerning this investigation. First, only plasma folate concentrations were determined as a measure of folate status. Secondly, the authors did not estimate folate intakes from the milk feedings or solid foods which would have a definite influence on folate nutriture. Lastly, the milk folate content was not determined due to "technical difficulties". The latter point could be of critical concern since recommendations have been based on the amount of the vitamin in breast milk. Recent studies have shown folate concentrations in milk in the range of 80-140 $\mu\text{g/L}$ (Smith et al, 1983; Tamura et al, 1980) whereas earlier work estimated folate levels between 2-50 $\mu\text{g/L}$ (Matoth et al, 1965; Ramasatri, 1965; Burland et al 19). Thus a formula containing 35 μg folate/L may not provide adequate folate for this population.

Ek and Magnus (1979) studied 35 healthy breast fed infants for their first year of life and assessed growth and folate status as determined by plasma and red blood cell folate concentrations. At birth and throughout the period of

investigation they found that folate levels in both plasma and red cells were significantly higher than that of the adult reference group. From their results, Ek and Magnus concluded that the folate status of breast fed infants was optimal and suggested "that the optimal supply of the vitamin in artificial nutrition should be the amount of folate necessary to maintain plasma and red cell folate concentrations similar to those found in breast fed infants".

As well, Ek and Magnus (1980, 1982) investigated the plasma and red blood cell folate concentrations of infants fed homemade cow's milk mixtures and infants receiving two proprietary formulas (one containing 39 μg folate/L, the other 78 μg /L). They compared the folate status of these infants to that of breast fed infants previously studied (Ek and Magnus, 1979). They found folate deficiency in ten infants fed the cow's milk mixtures and attributed this deficiency to the loss of folate during the preparation of the formula. The infants receiving the formula with 39 μg folate/L had plasma and red cell folate levels significantly lower than the breast fed babies whereas those receiving the second formula (78 μg /L) had folate levels similar to those recorded in the breast fed group. Thus the authors concluded that the formula containing 78 μg folate/L provided adequate folate for full term infants. Ek and Magnus chose more appropriate parameters to establish folate status than Salmenpera's group but they also did not estimate folate intakes for their subjects.

Smith et al (1985) conducted a study to assess possible factors which may influence folate nutrition of infants (i.e. the type of milk, total folate intake, and iron). They determined the folate status (using serum and red cell folate concentrations plus total intake) of 67 full term infants from birth to one year of age. From their data, they found that during the first 3 months infants exclusively fed proprietary formula (160 μg folate/L) with or without added iron had blood folate levels 1.5 to 4 times higher than those infants fed human milk (85 μg /L) and adult controls. After the introduction of solid food, blood folate levels paralleled a decrease in milk folate intake and the folate concentrations were about the same in all groups. Smith and coworkers concluded that milk, regardless of the type, is an important source of folate during the first year of life and provides more than sufficient folate to maintain the blood folate content well above acceptable limits. However, folate deficiency has been reported in LBW infants who were fed formulas based on heated or boiled evaporated milk (Strelling et al, 1979) or pasteurized milk (Matoth et al, 1979).

The folate requirement for LBW infants should take into account birthweight, rate of growth, and maternal folate status. Determining folate requirements of LBW infants is crucial since there is a dramatic drop of blood folate levels after birth and an increased incidence of megaloblastic anemia (Herbert, 1987a). Megaloblastic anemia is mostly seen 3-8

weeks after birth and in those weighing less than 1700 g at birth (Rodriquez, 1980). Since folate is also required for DNA synthesis which is essential for cellular proliferation, rapidly growing LBW infants may need more folate per unit body weight than full term infants.

There has been much controversy over the need of folate supplementation for LBW infants. Stevens et al (1979) conducted a folate supplementation study on 246 LBW infants. One group received 100 μ g folate daily plus 10 mg elemental iron from the third week after delivery until 12 months of age. The second group received only the iron supplement for the same period of time. They observed that the median red blood folate levels remained within normal adult range in both groups of infants and that weight gain was not affected by folate supplementation. Thus Stevens and coworkers concluded that they did not have sufficient grounds to recommend folate supplementation for the first year of life.

Asfour et al (1977) studied fully intakes of 20 Lebanese infants. Folate intakes greater than 3.6 μ g/kg/d did not raise hemoglobin levels or improve weight gain. However, it was not stated if any of the infants were of low birthweight. On the other hand, Strelling et al (1966) found that in a group of LBW infants not given folate supplementation, 25% had megaloblastic anemia. It has been shown that a test dose of folate disappeared from the blood of LBW infants more rapidly than in older subjects without an increase in the urinary

output of the vitamin (Shojania and Hornaday, 1970). Some authors have interpreted this as an increase in utilization and suggested supplementation of 50-200 μg folate/d for this age group (Strelling et al, 1979).

Gandy and Jacobson (1977) studied the effect of folate supplementation on the growth of 17 erythroblastotic infants. They received either 2.5 or 5 mg of folate daily from about the sixteenth day of life until they were 3 months of age. Weight gain was followed for one year and compared to the weight gain of 34 erythroblastotic infants not receiving supplementation. By the end of the fourth month the treated group's median centiles for weight had risen from the 40th to the 80th. The untreated group's centile for weight rose as well but only to the 50th centile. During the second half of the year both groups' weight centiles decreased; to the 50th centile for the treated group and to the 25th for the untreated group. The investigators suggested that further work was needed to determine if a shortage of folate during the first year of life could lead to a decreased rate of weight gain and also to impaired higher functions in the central nervous system at a later age.

Could excess folate be potentially harmful to the growth and development of the LBW infant? Folate has been shown to have potent convulsive effects in experimental animals when injected directly into the cerebral spinal fluid or the brain (Hommes and Obbens, 1972). However, while studying folate

transport in the central nervous system, Spector and Lorenzo (1975) suggested that there may be an efficient homeostatic mechanism in the blood-brain barrier which probably helps maintain brain levels of folate within relatively narrow limits.

3.5 VITAMIN B₁₂ (COBALAMIN)

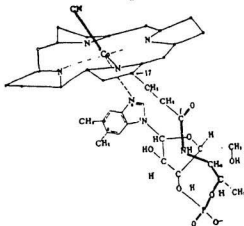
Vitamin B₁₂ (Figure 3.5) is defined as a group of cobalt-containing corrinoids that have biological activity in humans. Humans and other animals contain three main cobalamins; hydroxycobalamin, adenosylcobalamin, and methylcobalamin (Herbert, 1987b). Methylcobalamin is important because of its interaction with folate metabolism and deoxyadenosylcobalamin because of its involvement in both carbohydrate and lipid metabolism. Cyanocobalamin is the most widely used form of cobalamin in clinical practise due to its relative availability and stability. The conversion of cyanocobalamin to the coenzyme form is catalyzed by an enzyme system, vitamin B₁₂ coenzyme synthetase, present in a large number of microbes and human tissue.

Mammalian cells require cobalamin as a cofactor for two enzymes; (1) homocysteine methyltransferase which catalyzes the conversion of homocysteine to methionine, and (2) methylmalonyl coenzyme A mutase which catalyzes the rearrangement of methylmalonyl coenzyme A to succinyl coenzyme A. Vitamin B₁₂ is also involved in nucleic acid metabolism

through its role in the regeneration of folate from the 5-methyltetrahydrofolate pool and may influence the synthesis of polyglutamyl forms of folate (Ellenbogen, 1984; Herbert, 1987b; Shinton, 1972).

Figure 3.5

Structure of Cyanocobalamin



Absorption of vitamin B₁₂ takes place in the ileum either by an active or passive mechanism. Active transport is mediated by the intrinsic factor (IF), a mucopolysaccharide secreted by the parietal cells of the gastric mucosa. The cobalamin-IF complex attaches itself to specific receptors on the absorptive surface of the ileum (Ellenbogen, 1984). It has been recently hypothesized that the IF has two receptor sites; one for B₁₂ and one for the ileal intestinal microvilli (Herbert, 1987b). IF is not identified in plasma so it is presumed that an intestinal releasing factor splits it off and B₁₂ passes into the mucosal cell (Shinton, 1972; Herbert,

1987b).

Absorption may also occur by a passive mechanism which is nonIF mediated, and not dependent on the presence of calcium ions or pH. This process accounts for the absorption of 1-3% of the vitamin but becomes biologically significant when pharmaceutical amounts are consumed. This mechanism is site nonspecific and therefore, some absorption does occur through the nasal mucosa, rectal mucosa, and the skin (Shinton,1972).

It should be noted that as the intake of vitamin B₁₂ increases, there is a reduction in the percent of vitamin absorbed. At intakes of 0.5 µg or less, about 70% of the vitamin is absorbed. At intakes of 5 µg, the mean absorption is 28% (Herbert, 1987b).

The passage of cobalamin into the plasma begins approximately 2-3 hours after eating and peak plasma levels are attained after 8-12 hours. Most cobalamins are found in plasma attached to specific proteins, mainly transcorrin (transcobalamin) I, II, III (Ellenbogen, 1984; Shinton, 1972; Herbert, 1987b). The functional role of transcorrin I and III are unclear. While crossing the intestinal mucosa cobalamin is transferred to transcorrin II which facilitates the uptake of the vitamin by a large number of tissues. The binding of the transcorrin II-cobalamin complex to the cell surface is probably mediated by specific receptors which have not been identified. This binding is dependent on calcium ions, inhibited by EDTA and not temperature dependent (Ellenbogen,

1984). The transcobalamin II-cobalamin complex is degraded in the lysosome, freeing the cobalamin. Some of the cobalamin is used to synthesize methylcobalamin in the cytosol.

Many investigators have reported a total body cobalamin pool of 2-2.5 mg in adults and the normal turnover rate in humans is about 2.5 $\mu\text{g/d}$ (Heyssel et al, 1966; Shinton, 1972). In healthy individuals cobalamins are found principally in the liver. The vitamin B₁₂ concentration in the liver tends to increase with age (Hsu et al, 1966; McLaren, 1981). A three week old LBW infant was found to have a liver cobalamin content of 28 μg whereas an adult's liver content averages 1.5 mg (Ellenbogen, 1984). There are between 20-30 μg of the vitamin found in the kidneys, heart, spleen, and brain (Ellenbogen, 1984). The concentration of vitamin B₁₂ in plasma or serum is between 200-900 pg/mL with values less than 100 pg/mL indicative of a cobalamin deficiency.

The main excretory pathways for vitamin B₁₂ are through the urine, bile, and feces. The total loss from the body per day is about 0.1% of the body pool regardless of the pool's size (Heyssel et al, 1966). Urinary output of free cobalamin (the only form available for urinary excretion) varies from 0-0.25 $\mu\text{g/d}$ (Shinton, 1972; Ellenbogen, 1984). In pernicious anemia and other conditions associated with cobalamin deficiency, urinary excretion is reduced. Following TPN or intravenous administration, 50-90% of the dose is excreted in the urine within 48 hours (Ellenbogen, 1984). Patients with

renal disease excrete cyanocobalamin more slowly than healthy individuals (Ellenbogen, 1984).

About 0.5-5 μg of cobalamin per day is secreted into the alimentary tract, mainly in the bile. The ileum reabsorbs 65-75% of the vitamin through the IF mechanism. Small amounts of vitamin B₁₂ enter the intestine from gastric, pancreatic, and intestinal secretions. Unabsorbed cobalamin passes into feces and with that derived from bacterial synthesis in the colon could account for 3-6 μg daily depending on body stores (Ellenbogen, 1984; Herbert, 1987b).

Cobalamin stores in humans exceed the daily requirement 1000 fold and the small intestine contains microflora which can synthesize significant amounts of cobalamin (Ellenbogen, 1984). Therefore a clinical cobalamin deficiency due to dietary insufficiency is not often seen. It can take individuals consuming a vegan diet (void of animal products which is the main dietary source of vitamin B₁₂) 20-30 years to develop a deficiency (Herbert, 1987b). However, some reports (Jadhav et al, 1962; Higginbottom et al, 1978) have demonstrated a B₁₂ deficiency in infants breast fed by vegetarian mothers. The occurrence of severe cobalamin deficiency in infants whose nursing mothers' tissues are depleted of cobalamin indicates the importance of the vitamin to the growing child (Jadhav et al, 1962; Herbert, 1987b; Ellenbogen, 1984).

Inadequate absorption accounts for more than 95% of

vitamin B₁₂ deficiency cases. The lack of IF secretion results in the failure to absorb cobalamins and can result from pernicious anemia, gastrectomy, or the destruction of the gastric mucosa. It may take 5-7 years before any outward signs appear since body stores are usually sufficient for this length of time. Adult pernicious anemia is the most commonly acquired cause for failure to assimilate cobalamins. Pernicious anemia is an inherited autosomal dominant trait chiefly affecting persons past middle age. The incidence in the population is 1-2/1000. There is also a rare hereditary form of congenital pernicious anemia that clinically appears early in life. The symptoms of vitamin B₁₂ deficiency include megaloblastic anemia, weakness, tiredness, pale and smooth tongue, dyspnea, splenomegaly, leukopenia, thrombocytopenia, achlorhydria, paresthesia, neurological changes, loss of appetite, loss of weight, and low serum cobalamin levels.

A cobalamin deficiency may manifest in less than three years if a small intestinal defect develops from conditions such as celiac sprue, tropical sprue, resection of the ileum, or ileitis. In these cases there is no deficiency of IF but a defect in the ability of the distal ileum to absorb the cobalamin-IF complex despite its normal adsorption to the surface microvilli.

There are three known inborn errors of cobalamin metabolism. One results in a transcobalamin II deficiency causing megaloblastic anemia and other clinical symptoms early

in life (Cooper and Rosenblatt, 1987; Ellenbogen, 1984). Children respond well to weekly intramuscular injections of 1000 μg of cyanocobalamin. The other two are errors of tissue utilization characterized by a failure to synthesize one or both of the coenzymes leading to abnormal entry of cobalamins into cells. Megadoses of cyanocobalamin only partially correct this defect.

Vitamin B₁₂ appears to cause no toxicity in humans or animals at levels several thousand times their nutritional requirement (Herbert, 1987; Ek, 1987; Ellenbogen, 1984). Daily cobalamin requirements have been estimated from three types of studies; 1) the amount necessary to treat megaloblastic anemia from cobalamin deficiency, 2) comparison of blood and liver concentrations in normal and cobalamin deficient subjects, and 3) body stores and turnover rates of the vitamin. A dietary intake of 1 μg daily appears to sustain most healthy individuals (Herbert, 1987b). In fact, vegetarians require 1-1.5 μg orally to raise cobalamin levels and parenteral doses of about 1 $\mu\text{g}/\text{d}$ maintain patients with pernicious anemia in complete hematological and neurologic remission (Ellenbogen, 1984). To allow for variations in absorption and other factors, the RNI for adults was set at 2 μg (Health and Welfare Canada, 1983). The RNI for infants was derived from the content of vitamin B₁₂ in breastmilk and the amount of supplementation needed to show a therapeutic response to vitamin B₁₂ in deficient infants of B₁₂ deficient

vegetarian mothers (Herbert, 1987b; Jadhav et al, 1962). An oral intake of 0.3 $\mu\text{g}/\text{d}$ is recommended for infants according to the RNI for Canadians (Health and Welfare, 1983).

Vitamin B₁₂ deficiency is not seen in breast fed infants (apart from inborn errors of metabolism) unless their mothers are deficient. Jadhav et al (1962) examined 6 breast fed infants from South India with megaloblastic anemia. The abnormalities associated with this disorder were corrected by administering small doses of vitamin B₁₂ to these children which suggested that this was a specific effect of vitamin B₁₂ deficiency. In all cases, vitamin B₁₂ deficiency was attributable to either a dietary lack or malabsorption syndrome in the mothers. Higginbottom and coworkers (1978) studied a six month old infant who had been exclusively breast fed by a strict vegetarian mother. The clinical presentation of this infant (i.e. severe megaloblastic anemia, coma, and hyperpigmentation of the extremities) suggested an inborn error of metabolism and he was found to have methylmalonic aciduria and homocystinuria. Both the clinical and biochemical abnormalities resolved after therapy with vitamin B₁₂ supplementation. This case emphasized the importance of vitamin B₁₂ supplementation in breast fed infants of purely vegan women.

The common neonatal disorder, necrotizing enterocolitis, which can result in surgery with the resection of the terminal ileus may affect nutritional intake and metabolism of vitamin

B₁₂. Although much of the intestine has the ability to adapt its function after surgery, this does not occur for the absorption of vitamin B₁₂ which is dependent on the presence of specific receptor sites (Collins et al, 1984). Consequently, if there is inadequate vitamin B₁₂ absorption, vitamin B₁₂ deficiency will occur after hepatic stores have been exhausted. On the other hand, there are large liver stores of B₁₂ at birth (27 µg for term infants and a smaller reserve of 11 µg for LBW infants) (Vaz Pinto et al, 1975). Thus the vitamin B₁₂ content in newborns is rarely depleted prior to one year of age. However, there is increasing concern over vitamin B₁₂ deficiency in LBW infants since the developing brain is more susceptible to this deficiency than the developed one. An infant who is vitamin B₁₂ deficient may have neurological problems as well as megaloblastic anemia (Johnson et al, 1982).

Consequently, Collins and coworkers (1984) decided to assess the vitamin B₁₂ absorption in 14 children aged 1-7 who had ileal resections for necrotizing enterocolitis. All were born prematurely and none were given vitamin B₁₂ supplements. Six of these children had abnormal vitamin B₁₂ absorption, however, all had normal blood counts and serum vitamin B₁₂ concentrations. Despite this finding, these authors suggested that children with ileal resections should be on lifelong vitamin B₁₂ supplementation if they have abnormal vitamin B₁₂ absorption.

CHAPTER 4.0 PROBLEM STATEMENT

The vitamin requirements for LBW infants are uncertain. The aim of the present study was to determine the adequacy of thiamin, riboflavin, folate, and vitamin B₁₂ of LBW infants receiving 2 different levels of vitamins in their parenteral nutrition with that of a group of LBW infants receiving enteral feeds.

The present investigation was concerned with the adequacy of the intravenous infusion of four of the water-soluble vitamins; i.e. thiamin, riboflavin, folate and vitamin B₁₂ for the LBW infant. Thiamin and riboflavin play a role in neurological function. A deficiency of either may result in altered brain metabolism. It has been shown that 20% of infants born prematurely have minor or major neurological handicaps by the first year of life (Fitzharding et al, 1976). Furthermore, phototherapy for hyperbilirubinemia has resulted in riboflavin deficiency in this population group (Hovi et al, 1979). Folate is required for growth and erythropoiesis. At birth, LBW infants have limited folate reserves and are in a period of rapid growth. Thus they appear to have a increased need for this vitamin. Vitamin B₁₂ is a constituent of the coenzyme, methylcobalamin, which is involved in folate metabolism. Since there is a close interrelationship between folate and B₁₂ metabolism and because serum B₁₂ and folate can be measured simultaneously using a dual radioassay procedure,

it was convenient as well as practical to study vitamin B₁₂.

The objectives of this study were as follows:

- 1) to determine plasma levels of folate and vitamin B₁₂ and urinary thiamin, riboflavin, folate and vitamin B₁₂ concentrations of LBW infants receiving 2 levels of intravenous vitamin administration during parenteral nutrition; one level will be that level currently recommended (60% of the amount recommended for full term infants) and the other level will be 40% of the amount recommended for full term infants which still falls within the minimal advisable oral intakes for LBW infants (Table 4.1).
- 2) to determine erythrocyte transketolase and erythrocyte glutathione reductase activity, which are functional assays of thiamin and riboflavin status, respectively.
- 3) to measure the same parameters in infants receiving a commercially available formula designed for LBW infants.

TABLE 4.1

COMPOSITION-MVI-PEDIATRIC

	Minimal Advisable Oral Intake per day*	3 mL per day	2 mL per day
Thiamin (mg)	0.25-0.50	0.72	0.48
Riboflavin (mg)	0.34-0.68	0.84	0.56
Vitamin B ₁₂ (μg)	0.15-0.30	0.60	0.40
Folate (μg)	10-65	84.0	56.0

*Adapted from American Academy of Pediatrics, 1967; Fomon, 1974.

CHAPTER 5.0 METHODS

5.1 INTRODUCTION

Methods of vitamin analysis fall into three categories; animal, microbiological, and chemical assays. Animal assays determine vitamin availability to an animal using growth measurements and repletion tests. The principle disadvantages include length of time (i.e. 6-8 weeks for a growth test), cost, and variable results depending on the group of animals and techniques used, and interference from intestinal flora that can synthesize certain vitamins and confound results (Machlin, 1984; Augustyn et al, 1975).

Microbiological procedures include fermentation and growth of bacteria, yeasts, molds, or fungi. These methods consume less time, are less expensive and yield more reproducible results than animal assays. However, antibiotics and other substances (i.e. metabolites) may interfere with the interpretation of results (Machlin, 1984; Augustyn et al, 1985). Manual and automated chemical assays are accurate, rapid, and economical, and therefore, more applicable to routine determinations.

5.2 SUBJECTS

The study group consisted of 48 LBW infants allocated into three groups; parenterally fed infants (PAR2 and PAR3) and enterally fed infants (ENT). All LBW infants born at St.

Clare's Mercy Hospital or the Grace General Hospital after May 4, 1987 were eligible for the study if they weighed ≤ 1750 grams at birth, were free of congenital anomalies, and if parental consent was obtained. Consent was obtained once the study had been fully explained by either the nurse in the neonatal unit or by the investigator (appendix A and B). Subjects were removed from the study if they presented with low urine output (≤ 24 mL/kg/d) or they developed cholestatic jaundice (direct bilirubin more than 2.5 mg/100 mL).

The control group (ENT) consisted of 18 infants born at the Grace General Hospital who were receiving enteral feeds. The parenterally fed group of 30 infants were transferred to the Janeway Child Health Centre's Neonatal Unit within two days of birth. These infants received TPN and were randomly assigned to a group (PAR2) receiving 2 mL or 40% of the vial of the multivitamin supplement, MVI-Pediatric (McGraw Laboratories) or to a group (PAR3) receiving 3 mL or 60% of the vial. An infant was defined as "receiving TPN" if less than 25% of its energy intake was obtained from enteral feeds. Information regarding diagnosis, gestational age, birthweight, and weight throughout the study was collected from hospital records.

5.3 SAMPLE COLLECTION

5.3.1 BLOOD COLLECTION

Blood samples were collected prior to the initiation of enteral or parenteral feeds, when possible. Subsequent blood samples were collected at 7, 14, 21, and 28 days. All samples were collected by heel prick.

One millilitre (1 mL) of blood was collected in heparinized microtainers (Becton Dickinson) with one drop of sodium heparin added. One hundred microlitres (μ L) of whole blood was transferred to microtubes. The remaining blood was centrifuged at 1000 RPM for 10 minutes. The plasma was separated and transferred into polyethylene microtubes and frozen at -70°C . The red cells were washed with normal saline four to five times before dilutions were prepared. Two 50 μ L aliquots of red blood cells were diluted with 950 μ L of distilled water for the riboflavin assay and two 200 μ L aliquots of red blood cells were diluted with 200 μ L of distilled water for the thiamin assay. All samples were then frozen at -70°C until analysis.

LBW infants may receive blood transfusions for four reasons; 1) to replace acute blood loss, 2) to replace chronic blood loss, 3) to treat hypotension, and 4) to reverse the chronic anemia sometimes seen at 7 to 8 weeks of age (Joshi et al, 1987). Therefore, ten samples of transfused blood were analyzed for thiamin, riboflavin, and folate

concentrations.

5.3.2 URINE COLLECTION

Twenty-four hour urine collections were obtained prior to the initiation of enteral or parenteral feeds, when possible. Subsequent urine samples were collected at 7, 14, 21, and 28 days.

Initially urine collecting bags (Hollister U-Bags) for female infants and 10 mL polyethylene tubes for the males were used for urine collections. The success of these methods depends on constant observation and prompt emptying which was not feasible in this study. Consequently, (especially with respect to the girls) more urine was lost than acquired and only rough estimations of total urinary output volumes could be made.

A collection method that would not interfere with patient care and would allow for a more realistic 24 hour output volume was needed. A "metabolic" bed, suitable for an isolette, was modified from one that was in the possession of Dr. Wayne Andrews, Neonatologist at the Janeway Child Health Centre. The frame of the bed was made of plexiglass (Appendix C) and is 53.4 x 30.5 x 7.7 cm. Bed sheets were made from Swedish Nitex Mesh (Appendix D) allowing urine to seep through to the collecting pan and excluding the feces. The mesh sheet sometimes caused skin irritation and therefore elbows and knees of these infants were covered with gauze. Urine was

collected in brown Nalgene bottles and stored at -70°C until analyzed.

5.4 VITAMIN ANALYSIS OF BLOOD SAMPLES

5.4.1 THIAMIN

Both blood and urinary excretion of thiamin are only a reflection of the immediate intake and may not be a reliable index of tissue stores, distribution, or actual biochemical functioning (Saubert et al, 1974). Thus these values may be of limited value for the interpretation of actual thiamin status. In controlled human studies (Saubert et al, 1974) a relationship has been found to exist between thiamin intake, urinary excretion, and erythrocyte transketolase activity.

In the present study the transketolase stimulatory effect or the thiamin pyrophosphate (TPP) effect, a functional assay developed by Brin et al (1965), was used to determine thiamin status. Transketolase, a TPP dependent enzyme, catalyzes the following two reactions in the pentose phosphate pathway:

- (1) Xylulose-5-phosphate + ribose-5-phosphate $\xrightleftharpoons{\text{Transketolase-TPP}}$ sedoheptulose-7-phosphate + Glyceraldehyde-3-phosphate
- (2) Xylulose-5-phosphate + erythrose-4-phosphate $\xrightleftharpoons{\text{Transketolase-TPP}}$ fructose-6-phosphate + Glyceraldehyde-3-phosphate

Transketolase is found within red blood cells and is independent of changes in extracellular plasma (Brin et al, 1965). As thiamin deficiency becomes more severe, thiamin becomes limiting in the body cells and the available TPP becomes depleted resulting in a decrease of transketolase activity. Thus the TPP effect measures the extent of depletion of the transketolase enzyme.

Hemolyzed red blood cell (RBC) samples were incubated in a buffered medium with an excess of substrate (ribose-5-phosphate). The tubes were preincubated for 30 minutes either in the presence or absence of excess TPP (Brin et al, 1965). The supernatant was prepared by following the incubation chart (Table 5.1). Following an incubation period of 60 minutes at 37°C, the tubes were centrifuged for 10 minutes and the supernatant was saved for determining the amount of pentose utilized. During the first incubation period, 100 μ L of substrate was added to produce an equilibrium mixture of ribose-5-phosphate, ribulose-5-phosphate, and xylulose-5-phosphate. The remaining pentose was measured in the supernatant (Table 5.2). These compounds are converted to furfural and furfural derivatives by concentrated HCl and the condensation of a polyhydric phenol (orcinol) in the presence of metal ions (Fe^{++}) to form colour complexes. These compounds have absorption maxima at 670 nm and the absorbances were read against a blank set at zero in a Perkin Elmer 200 spectrophotometer.

TABLE 5.1
ERYTHROCYTE TRANSKETOLASE ACTIVITY (TPPE %)
INCUBATION CHART

TUBES	HEMOLYSATE (mL)	BUFFER (mL)	TPP (mL)	INCUBATE	SUBSTRATE (mL)	INCUBATE	5% TCA (mL)
A	0.1	0.4	- V	30	0.1 V	60	3 V
			O		O		O
B	0.1	-	0.4 R	30	0.1 R	60	3 R
			T		T		T
D	0.1	0.5	- E		- E		3 E
			X		X		X
R	-	0.5	-		0.1		3

TABLE 5.2
ERYTHROCYTE TRANSKETOLASE ACTIVITY (TPPE X)
PENTOSE UTILIZATION DETERMINATION

TUBES	FILTRATE (mL)	PENTOSE (mL)	DISTILLED WATER (mL)	ORCINOL (mL)	BOILING WATER BATH	COLD ICE BATH
A	0.2	-	1.3	4.5 V	All	All
B	0.2	-	1.3	4.5 R	tubes	tubes
D	0.2	-	1.3	4.5 T	for 20	for 5
R	0.1	-	1.4	4.5 E	min.	min.
				X		
STD5	-	0.5	1.0	4.5		
STD10	-	1.0	0.5	4.5		
BLANK	-	-	1.5	4.5		

Orcinol was added at 1 minute intervals to each tube
All tubes are read against a blank set at zero at 670 nm

The concentrations of these derivatives are a measure of transketolase activity. The TPP effect reflects the proportion of transketolase which was not saturated with TPP. The amount of pentose utilized per mL hemolysate per hour was calculated using the calculations outlined in Appendix E.

An increase in enzyme activity resulting from added TPP is referred to as the TPP effect. Values obtained without TPP represent absolute enzyme activity and are dependent upon the enzyme available in the erythrocytes. Addition of TPP permits an estimation of the amount of apoenzyme uncomplexed as well as of the maximum potential transketolase activity. A TPP effect of greater than 15% is indicative of a thiamin deficiency (Brin et al, 1965). A severe deficiency is demonstrated in humans with a TPP effect of 25% or more (Saubertlich, 1965).

5.4.2 RIBOFLAVIN

The functional test (Tillotson and Baker, 1972), erythrocyte glutathione reductase (EGR) activity, was used to assess the nutritional adequacy of riboflavin. EGR catalyzes the reduction of oxidized glutathione (GSSG):



This assay is performed spectrophotometrically requiring only small quantities of blood and is based on the determination

TABLE 5.3
ERYTHROCYTE GLUTATHIONE REDUCTASE ACTIVITY
ASSAY MEDIA

REAGENT	BLANK	CUVETTE 1	CUVETTE 2
Buffer (ml)	2	2	2
EDTA (μ L)	50	50	50
GSSG (μ L)	100	100	100
WATER (μ L)	200	-	100
FAD (μ L)	-	100	-
HEMOLYSATE (μ l)	-	100	100

of the activity of EGR in the presence and absence of added coenzyme, FAD. The degree of stimulation resulting from the in vitro addition of FAD is expressed in terms of the activity coefficient (AC) (Appendix F).

The assay medium was prepared in duplicate as outlined in Table 5.3. The medium was incubated at 37°C for 8 minutes in the cuvette chamber of a Perkin Elmer 200 spectrophotometer. Following incubation, 100 µL of NADPH was added to each cuvette. The absorbance of NADPH at 340 nm was measured for 5 minutes.

The AC is calculated using the following equation:

$$AC = \frac{\text{Reduction of absorbance of NADPH with FAD}}{\text{Reduction of absorbance of NADPH without FAD}}$$

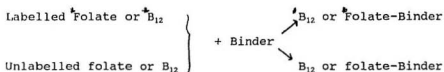
Normal subjects have an AC equal to 0.9 to 1.2 indicating little or no stimulation. In riboflavin deficiency a marked stimulation is observed and this is associated with a decreased urinary output of ribcflavin (Tillotson and Baker, 1972).

The AC is age and sex independent whereas there are age and sex differences encountered with urinary excretion of riboflavin. Animal studies provide evidence that deficiencies of other vitamins such as thiamin, folate, pyridoxine, and vitamin C along with a decreased caloric intake do not invalidate the interpretation of the FAD effect (Sauberlich et al, 1974). Uremia and cirrhosis of the liver may compound

interpretations (Tillotson and Baker, 1972; Selhub and Rosenberg, 1984).

5.4.3 FOLATE AND VITAMIN B₁₂

A dual radioassay kit purchased from Becton Dickinson (SimulTRAC-S) was used to analyze both RBC folate and plasma folate concentrations as well as plasma vitamin B₁₂ levels. The basis of this assay is competitive protein binding as explained by the following equation:



Unlabelled vitamin competes with labelled vitamin for the limited number of binding sites and thus reduces the amount of labelled vitamin bound. Consequently, the level of radioactivity bound is inversely related to the concentration of the vitamin in the serum or plasma.

The folate binder included in this kit is derived from milk. The pteroylglutamic acid form of folate (PGA) is used for the standards and tracer in an incubation mixture at pH 9.3. Both PGA and 5-methyltetrahydrofolic acid (MTFA) have equal affinity for the solid phase milk binder at pH 9.3 but PGA is more stable in solution than MTFA.

For vitamin B₁₂ the binder is purified intrinsic factor (porcine) which is specific for cobalamin. The intrinsic

factor and the folate binder are covalently linked to a solid support.

Polyethylene microtubes were numbered for standards and samples according to Table 5.4. This assay commences by destroying endogenous serum binders through heat denaturation (boiling for 15 minutes). After boiling, a constant amount of binder is added. The tubes are then incubated at room temperature for 60 minutes to allow the competitive binding reaction to take place. The separation of bound and free folate and vitamin B₁₂ is achieved by centrifugation. The supernatant (free molecules) is then decanted and the bound (pellets) are counted for one minute with a scintillation (gamma) counter (Beckman Gamma 5500).

From the calculations described in appendix G, a standard curve is plotted on logit-log paper (appendix H). The concentration of vitamin B₁₂ or folate in the samples can be obtained from the standard curve and corrected by the dilution factor.

The normal value for humans for plasma folate is 2-45 ng/mL and for RBC folate >110 ng/mL (Becton Dickinson, 1982). Values lower than these are indicative of a folate deficiency (Herbert, 1987a). For plasma vitamin B₁₂ the normal range is between 200-900 pg/mL. Serum vitamin B₁₂ levels well above 1000 pg/mL are suggestive of either liver disease or myeloproliferative disorders (Herbert, 1987b).

TABLE 5.4
RADIOASSAY-FOLATE AND VITAMIN B₁₂ PROCEDURE

TUBE	STD μL	SAMPLE μL	WORKING TRACER SOLUTION μL	INCUBATE	BLANK REAGENT μL	BINDER μL	INCUBATE	CENTRIFUGE
1,2	-	-	1000	-	-	-	-	-
3,4	200A*	-	1000	-	200	-	-	-
5,6	200A	-	1000	At	-	200	At	At
7,8	200B	-	1000	100°C	-	200	room	1000g
9,10	200C	-	1000	for	-	200	temp	(3.5
11,12	200D	-	1000	15 min.	-	200	for	min ⁻¹)
13,14	200E	-	1000	Cool	-	200	60	for
15,16	200F	-	1000	to	-	200	min.	10 min
17,18	-	200	1000	18-25°C	-	200	in	(in
etc.	-	200	1000	-	-	200	dark	cold)

*A,B,C,D,E,F refer to standards of differing concentrations of folate and vitamin B₁₂ provided in the kit.

5.5

VITAMIN ANALYSIS OF URINE SAMPLES

5.5.1 THIAMIN

To determine urinary thiamin, a modified thiochrome procedure was used (Leveille, 1972). Thiamin can be oxidized to thiochrome by alkaline potassium ferricyanide. The fluorescence of thiochrome can then be measured. The main problem with this method has been the presence of unidentified fluorescing substances found in varying amounts. This problem has been solved (Leveille, 1972) by either isolating thiamin or by using a blank to correct for the fluorescence due to these unidentified substances. The fluorescence measured is proportional to the concentration of thiochrome and thus to the amount of thiamin in the sample.

This assay uses a 3 tube method to determine urinary thiamin excretion as outlined in Table 5.5. The first tube (A) contained the thiamin standard which corrected for any quenching of fluorescence produced by the individual urine sample. Tube B contained the urine sample to be measured and tube C was the blank. The blank was prepared by adding sodium hydroxide and benzene sulphonyl chloride to the sample in order to block the oxidation of thiamin to thiochrome.

The isobutanol layer was transferred to fluorometer tubes and fluorescence was then measured using a Perkin-Elmer LS-5 Spectrofluorometer. The excitation wavelength was 365 nm and the emission wavelength 435 nm. The excitation slit width was

TABLE 5.5
URINARY THIAMIN ASSAY (THIOCHROME PROCEDURE)

REAGENTS	TUBE	TUBE	TUBE	COMMENTS
	A	B	C	
URINE (mL)	2	2	2	Vortex all tubes and wait at least 1 min
THIAMIN (mL) (0.2 µg/mL)	2	-	-	
WATER (mL)	1	3	2	
1 N NaOH (mL)	-	-	1	
*BSC (drops)	-	-	5	
**OXIDIZING REAGENT	2.5	2.5	2.5	Vortex and wait 50-60 sec.
H ₂ O ₂ (30%) (drops)	2	2	2	Vortex
ISOBUTANOL (mL)	10	10	10	Cap and vortex

*Benzene Sulphonyl Chloride

**Oxidizing Reagent: 2 parts 2% potassium ferricyanide to 4 parts 40% NaOH

5 nm and the emission slit width was 5 nm for baseline urines and slit widths of 5 nm and 3 nm were used for all other samples, respectively. For reagent preparation and calculations refer to appendix I.

5.5.2 URINARY RIBOFLAVIN (FLUOROMETRIC APOPROTEIN TITRATION METHOD)

In solution individual proteins fluoresce as do flavins which have a separate and distinct fluorescent spectrum. When flavins bind with equimolar quantities of their respective apoprotein the fluorescence of both the flavins and protein are almost completely quenched. This quenching of fluorescence makes possible the direct titration of the apoprotein with flavin which is the basis of this assay (Tillotson and Bashor, 1980).

Riboflavin fluorescence is quenched when it binds egg white flavoprotein. The concentration of urinary riboflavin is calculated from the difference between the fluorescence before and after the apoprotein titration.

Fluorescence was determined in a Perkin-Elmer LS-5 spectrofluorometer. The excitation wavelength was 450 nm and the emission wavelength was 520 nm. The excitation slit width was 5 nm and the emission slit width was 20 nm for all samples. The standard or sample was transferred to the cuvette and an initial fluorescence was obtained. Then 10 μ L of apoprotein was added and the fluorescence recorded. Ten

(10) μL aliquots of apoprotein were added until the same fluorescence reading was obtained after three consecutive additions of apoprotein. A standard curve of the change in fluorescence units against the riboflavin concentration was plotted. The concentration of urinary riboflavin was calculated from the standard curve. For reagent preparation refer to appendix J.

5.5.3 FOLATE AND VITAMIN B_{12}

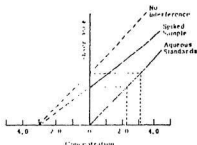
There is no assay currently available for determining the urinary excretion of folate and vitamin B_{12} . Therefore, the radioassay, the method used to determine blood levels, was carried out to assess its adequacy for determining urinary excretion levels using the Method of Standard Additions (Beatty, 1978) (Figure 5.1).

Accurate determinations can be made without eliminating interferences by making the concentration calibration in the presence of the interference. The standards are added to portions of the sample, thereby letting any interferent present in the sample also affect the standard.

The urinary methylmalonic acid test is a well established method used to diagnose cobalamin deficiency (Chalmers and Lawson, 1982). Twelve urine samples (4 from each study group) were analyzed by the Janeway Child Health Centre's laboratory for methylmalonic acid. Methylmalonic acid was measured quantitatively by gas chromatography mass spectrometry using

Figure 5.1

Method of Standard Additions*



Adapted from Concepts, Instrumentation and Techniques in Atomic Absorption Spectrophotometry

R.D. Beatty, Perkin Elmer Corporation, 1978

A sample is taken and split into 3 portions. Nothing is added to the first portion; different amounts of standard are added to portions 2 and 3. Finally, all portions are diluted to the same volume so that the final concentrations are the same. Only the amount of added analyte differs by a known amount. If there is no interference in the sample, then the final concentration vs concentration of the added standard would be parallel to the aqueous standard calibration. If there is interference, the amount of the analyte in the unspiked sample will be reduced by this interference. However, the increase from added standard will also be reduced by the same amount. Therefore, a straight line will result but because of the interference, its slope will be different from that of the aqueous standards. By continuing the concentration calibration on the abscissa backward from zero and extrapolating the calibration line backward until it intercepts the concentration axis, the concentration of the unspiked sample can be determined.

selective ion monitoring (Chalmers and Lawson, 1982).

5.6 DIETARY INTAKE DATA

Energy, thiamin, riboflavin, folate, and vitamin B₁₂ intakes were calculated from product monographs. Due to the multidisciplinary nature of neonatal intensive care for LBW infants, not all the prepared TPN solutions were infused over a 24 hour period at certain times. Therefore, the infants in the parenteral groups did not always receive their assigned 2 mL or 3 mL of the multivitamin supplement.

5.7 PARENTERAL NUTRITION INFUSATES

Three 200 mL samples of parenteral nutrition solutions were exposed to normal conditions in the Neonatal Unit at the Janeway Child Health Centre for a 30 hour period. For the first 12 hours aliquots were removed every 2 hours starting with time zero and thereafter, every 4 hours. An equal amount of 0.1 N HCl was added to the vial containing the TPN mixture to be used for determining thiamin concentration for the purpose of stabilization. The samples were protected from light and frozen immediately upon removal from the TPN bags at -20°C. The samples were analyzed for the thiamin, folate and B₁₂ levels following the procedures outlined in section 5.5.1, and 5.5.2, respectively. As well, MVI-Pediatric was analyzed for these 3 vitamins using the same analytical techniques.

Riboflavin is present as FMN in MVI Pediatric and therefore, in the TPN solutions. The fluorometric apoprotein procedure used in this study for determining urinary riboflavin is specific for riboflavin and only binds loosely with FMN. Since no technique was available to analyze FMN in this laboratory, riboflavin was not measured in the vial of MVI-Pediatric or TPN admixture.

5.8 STATISTICAL ANALYSIS

All data were entered onto the VAX computer system. Because subjects were placed on enteral feeds once clinically indicated, subjects dropped out of the PAR2 and PAR3 groups rapidly. Therefore, changes in vitamin levels could not be analyzed using the repeated measures analysis of variance. To evaluate if there was an effect of time on each variable within groups, a statistical formula (Appendix K) was designed specifically for this problem by Dr. Sutradhar, Department of Mathematics and Statistics, Memorial University of Newfoundland. This procedure and oneway analysis of variance was used to assess differences between groups at each sampling time for each vitamin. Oneway analysis of variance was also used to determine differences due to birthweight and gestational age. Students t-test was used to determine if there was a difference due to gender or acquiring blood transfusions within each group. Oneway analysis of variance and student T-tests were done by SPSSX computerized

procedures. Linear regression was used to determine if there was a correlation between dietary thiamin or riboflavin and urinary output of these two vitamins as well as dietary folate and vitamin B₁₂ and their plasma concentrations.

6.1 INTRODUCTION

Demographic information regarding gestational age, birthweight, and sex is presented in Table 6.1. There was no significant difference in gestational age or birthweight between the three study groups. No effect due to gender differences was observed within the groups for each biochemical parameter examined.

TABLE 6.1
DEMOGRAPHIC INFORMATION ($\bar{X} \pm SD$)

	ENT	PAR 2 mL	PAR 3 mL
NUMBER OF SUBJECTS	18	15	15
BIRTHWEIGHT (g)	1281 \pm 271	1137 \pm 231	1133 \pm 293
GESTATIONAL AGE (WEEK)	29.6 \pm 1.7	28.5 \pm 1.8	29.4 \pm 2.1
SEX			
FEMALE	9	11	3
MALE	9	4	12

The two major prematurity related medical problems affecting these infants were respiratory distress syndrome and hyperbilirubinemia. Fifty per cent of the ENT infants, 80% of the PAR2 group and 100% of the PAR3 group were diagnosed as having respiratory distress syndrome. Hyperbilirubinemia was clinically presented in 61% of the ENT group, 13% of the PAR2 group, and 60% of the PAR3 group. Seventy-nine per cent of the infants received an antibiotic and 69% received theophylline for apnea of prematurity as part of their medical treatment during the study period. Forty-eight per cent of the infants underwent phototherapy, the treatment for hyperbilirubinemia, in their first week of life.

Only 50% of the PAR2 and PAR3 infants were still receiving parenteral nutrition by the beginning of the second week and by the beginning of the third week less than a third of the infants who started the study received TPN. Some infants in the parenteral groups failed to complete the study because of concurrent illness involving additional treatment or because of their progression to complete enteral feeds. During the course of this investigation one child in the PAR3 group died. Because there were few samples, if any, available by week 3, parameters measured at this week were not included on the graphs. However, they are listed in the tables provided. Not all the prepared feedings were infused over a 24 hour period. Table 6.1a shows the actual percentage of parenteral fluid intake which was achieved for the PAR2 and

PAR3 groups. From these results it appears that the PAR2 group only received approximately 1.5 mL/d of MVI-Pediatric.

Twenty per cent of the infants received blood transfusions at some point in the study. No differences in erythrocyte glutathione reductase activity, erythrocyte transketolase activity, or RBC folate concentrations were found between those who received transfusions and those who did not. Thus, the values were pooled.

Body weight was used to assess growth. No significant difference occurred between the 2 parenteral groups at any sampling time throughout the study period, suggesting that the different levels of vitamins infused did not influence growth. However, the subjects in the ENT group were approximately 300 g larger than the subjects in the parenteral groups by the second week (Figure 6.1). The ENT infants were significantly larger than the PAR3 infants at the 0.05 level of significance.

There was no significant difference in the amount of energy received by either the PAR2 or PAR3 groups (Figure 6.2). On the other hand, the ENT infants received twice as much energy as the infants receiving TPN by the second week ($p < 0.05$). The mean percentage of energy and vitamins provided by enteral feeds for the parenteral groups was only 4% of the total. Consequently, the enteral intake was a minor contributor of these nutrients.

TABLE 6.1a

PERCENT (%) TPN RECEIVED BY PARENTERAL GROUPS
($\bar{X} \pm SD$) (n)

	PAR2	PAR3
Week 1	76 \pm 26 (13)	71 \pm 47 (13)
Week 2	78 \pm 18 (6)	91 \pm 10 (7)
Week 3	82 \pm 12 (3)	64 \pm 22 (4)

WEIGHT VS TIME

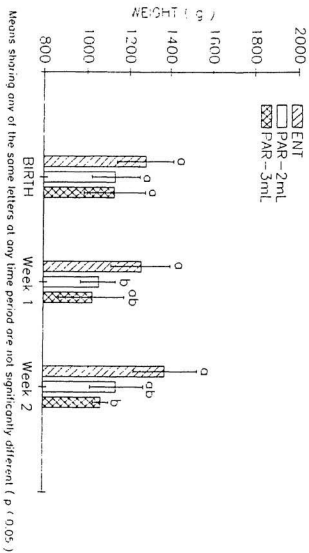
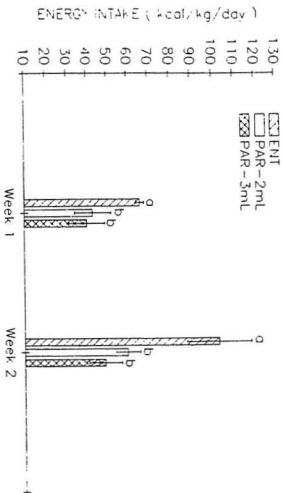


Figure 6.1

ENERGY INTAKE VS TIME



Means sharing the same letters at any time period are not significantly different ($p < 0.05$)

Figure 6.2

6.2 VITAMIN CONCENTRATIONS IN TPN ADMIXTURES AND MVI-PEDIATRIC

Table 6.2 lists the vitamin concentration in three TPN solutions exposed to normal conditions in the neonatal unit at the Janeway Child Health Centre over a 30 hour period. There was no degradation in the thiamin level in TPN solutions over the 30 hour period. The actual amount measured was, on average, 38% more than the level expected. The thiamin concentration in MVI-Pediatric was found to be 35% more than the amount listed on the product monograph. Due to this result, calculated intakes were adjusted by 38% for the parenteral groups (Figure 6.3, Table 6.3) and used for the interpretation of thiamin status in this investigation.

The folate concentration in the TPN admixtures declined by 25% after 24 hours. However, the level of folate detected (.028 mg folate/mL MVI-Pediatric) in the parenteral solution at time 0 and in the vial of MVI-Pediatric (Table 6.2) was the concentration expected. Intakes were calculated using the amount of folate appearing on the package insert of MVI-Pediatric for the PAR2 and PAR3 groups.

Vitamin B₁₂ in the parenteral solution was stable over time (Table 6.2). The amount of vitamin B₁₂ recovered in the multivitamin formulation was 90% of the level expected. Thirty-three percent less vitamin B₁₂ was measured in the TPN admixture at time 0 than the level expected. In this study, intakes of vitamin B₁₂ by the parenteral groups were adjusted

TABLE 6.2

VITAMIN CONCENTRATIONS IN TPN ADMIXTURE AND MVI-PEDIATRIC

	THIAMIN (mg/mL)	FOLATE (mg/mL)	VITAMIN B ₁₂ (μg/mL)
TPN-Time 0 h (admixture)	0.330±0.123	0.028±0.0	0.133±0.009
TPN-Time 24 h (admixture)	0.370±0.093	0.021±0.001*	0.133±0.008
TPN-Time 30 h (admixture)	0.310±0.030	0.021±0.002*	0.133±0.004
MVI-PEDIATRIC	0.330±0.001	0.028±0.0	0.180±0.005
PRODUCT MONOGRAPH	0.240	0.028	0.200

*Significantly different than Time 0 at the 5% level.

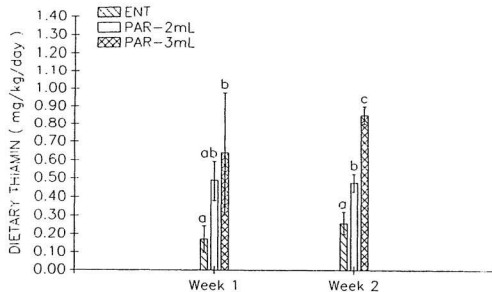
downward by 33%.

6.3 THIAMIN

Figure 6.3 and Table 6.3 show the dietary thiamin intakes for each group. Two millilitres of MVI-Pediatric added to the parenteral solution supplied 0.48 mg of thiamin daily whereas 3 mL supplied 0.72 mg. The PAR3 infants received significantly more thiamin than the infants in the ENT group in the first and second weeks. The PAR2 infants received more of the vitamin than the ENT infants by week 2. None of the infants had biochemical evidence of a thiamin deficiency as measured by the thiamin pyrophosphate effect (TPPE) (Figure 6.4, Table 6.4). There was no significant difference between groups at any time period with respect to the TPPE but there was a time effect found between the baseline and week 1 results for all three groups. No difference was seen between the 3 groups at weeks 1 through 3 regarding transketolase activity (Table 6.4a). The TPPE was within the normal range for the 10 transfused blood samples ($4\% \pm 3\%$).

Urinary thiamin levels (Figure 6.5, Table 6.5) were significantly lower prior to the initiation of parenteral or enteral feeds than at subsequent sampling times. However, there was no difference found between groups during the study period. Mean daily thiamin intake exceeded mean daily thiamin excretion in weeks 1 through 3 resulting in net positive balance (Table 6.6). However, it is noteworthy that 27%, 45%,

DIETARY THIAMIN vs TIME



Means sharing the same letters at any time period are not significantly different ($p < 0.05$)

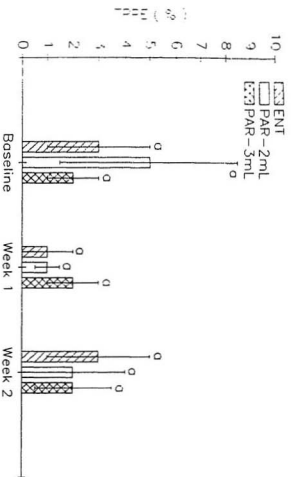
Figure 6.3

TABLE 6.3
DIETARY THIAMIN (mg/kg/d) $\bar{X} \pm SD$ (n)

	ENT	PAR2	PAR3
Week 1	0.171 \pm 0.143* (15)	0.487 \pm 0.211 ^{ab} (12)	0.640 \pm 0.775 ^b (13)
Week 2	0.259 \pm 0.121* (17)	0.476 \pm 0.093 ^b (7)	0.848 \pm 0.104 ^c (6)
Week 3	0.271 \pm 0.053* (15)	0.520 \pm 0.170 ^b (4)	0.653 \pm 0.306 ^b (3)

Means sharing the same letters in a row at any time periods are not significantly different ($p < 0.05$).

RBC TPPE VS TIME



Means sharing the same letters at any time period are not significantly different ($p < 0.05$)
Figure 6.4

TABLE 6.4

ERYTHROCYTE TRANSKETOLASE ACTIVITY (TPPE %) $\bar{X} \pm SD$ (n)

	ENT	PAR2	PAR3
Baseline	3±4 ^a (11)	5±7 ^a (14)	2±2 ^a (11)
Week 1	1±2 ^a (12)	1±3 ^a (10)	2±2 ^a (9)
Week 2	2±3 ^a (7)	2±4 ^a (6)	2±3 ^a (5)
Week 3	2±2 (7)	-	1±1 (2)

Means sharing the same letters in a row at any time periods are not significantly different ($p < 0.05$).

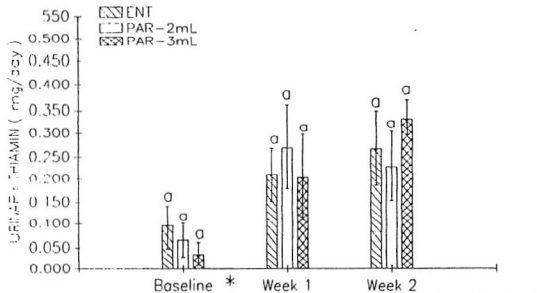
TABLE 6.4a

TRANSKETOLASE ACTIVITY
(mg of pentose/mL hemolysate/h) $\bar{X} \pm SD$ (n)

	ENT	PAR2	PAR3
Baseline	3.46 \pm 0.64 ^a (13)	2.90 \pm 0.35 ^b (14)	3.06 \pm 0.49 ^{ab} (11)
Week 1	3.35 \pm 0.50 ^a (12)	3.00 \pm 0.29 ^a (5)	3.15 \pm 0.23 ^a (5)
Week 2	3.28 \pm 0.42 ^a (7)	2.84 \pm 0.42 ^a (3)	3.46 \pm 0.05 ^a (3)
Week 3	3.51 \pm 0.51 (6)	-	3.38 (1)

Means sharing the same letters in a row at any time periods are not significantly different ($p < 0.05$).

URINARY THIAMIN vs TIME



Means sharing the same letters at any time period are not significantly different ($p < 0.05$)

* A significant difference between baseline and the other time periods was observed within groups ($p < 0.05$)

Figure 6.5

TABLE 6.5
URINARY THIAMIN (mg/d) $\bar{X} \pm SD$ (n)

	ENT	PAR2	PAR3
Baseline*	0.095 \pm 0.094* (7)	0.065 \pm 0.072* (8)	0.035 \pm 0.052* (7)
Week 1	0.210 \pm 0.117* (13)	0.269 \pm 0.184* (12)	0.230 \pm 0.187* (12)
Week 2	0.243 \pm 0.175* (11)	0.232 \pm 0.193* (6)	0.333 \pm 0.074* (5)
Week 3	0.278 \pm 0.178* (8)	0.315 \pm 0.060* (2)	0.342 \pm 0.258* (3)

*A significant difference was observed between baseline values and levels at subsequent weeks for all 3 groups ($p < 0.05$).

Means sharing the same letters in a row at any time periods are not significantly different ($p < 0.05$).

TABLE 6.6
THIAMIN BALANCE $\bar{X} \pm SD$ (n)

Group	Intake mg/kg/d	Output mg/kg/d	%Intake Excreted	Net Retention mg/kg/d
ENT				
week 1 (11)	0.198 \pm 0.147	0.175 \pm 0.095	88	0.023 \pm 0.149
week 2 (11)	0.268 \pm 0.124	0.188 \pm 0.136	70	0.080 \pm 0.177
week 3 (11)	0.268 \pm 0.073	0.202 \pm 0.137	75	0.067 \pm 0.149
PAR2				
week 1 (11)	0.485 \pm 0.222	0.249 \pm 0.160	50	0.236 \pm 0.314
week 2 (6)	0.482 \pm 0.101	0.222 \pm 0.177	46	0.261 \pm 0.199
week 3 (2)	0.550 \pm 0.195	0.275 \pm 0.096	50	0.275 \pm 0.136
PAR3				
week 1 (10)	0.658 \pm 0.791	0.110 \pm 0.053	17	0.549 \pm 0.806
week 2 (4)	0.858 \pm 0.079	0.342 \pm 0.070	40	0.516 \pm 0.107
week 3 (3)	0.495 \pm 0.191	0.343 \pm 0.258	69	0.152 \pm 0.053

and 33% of the ENT, PAR2, and PAR3 infants respectively were in negative thiamin balance in week 1 and by week 2 the proportions were 27%, 17%, and 0%. There was no correlation found between dietary intake and urinary excretion for all the groups at any time period.

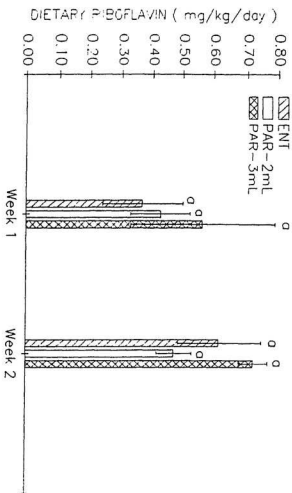
6.4 RIBOFLAVIN

Dietary riboflavin, erthrocyte glutathione reductase (EGR) activity, and urinary output for the three groups are shown in figures 6.6, 6.7, and 6.8, respectively, and in Tables 6.7, 6.8, and 6.9. Two millilitres of MVI-Pediatric supplied .56 mg of riboflavin daily whereas 3 mL supplied .84 mg. Figure 6.6 illustrates the actual calculated riboflavin intake for each group. Since not all the prepared feedings were infused into the infants receiving TPN, there was no significant difference between the levels of riboflavin provided to the 3 study groups.

The EGR activity was within the normal range for all groups at each time period. However, the EGR activity differed significantly between the PAR3 and PAR2 groups at week 2 (Figure 6.7, Table 6.8). The EGR activity for 10 samples of transfused blood was within the normal range ($1.04 \pm .15$).

Urinary riboflavin output (Figure 6.8, Table 6.9) followed the same trend as was seen with urinary thiamin concentrations. The riboflavin levels at baseline within each

DIETARY RIBOFLAVIN VS TIME



Means sharing the same letters at any time period are not significantly different ($p < 0.05$)

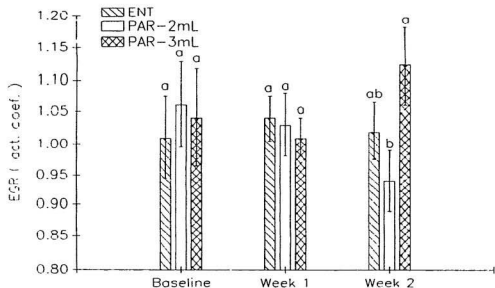
Figure 6.6

TABLE 6.7
DIETARY RIBOFLAVIN (mg/kg/d) $\bar{X} \pm SD$ (n)

	ENT	PAR2	PAR3
Week 1	0.368 \pm 0.263* (16)	0.427 \pm 0.191* (13)	0.561 \pm 0.656* (13)
Week 2	0.617 \pm 0.264* (16)	0.471 \pm 0.114* (7)	0.721 \pm 0.087* (7)
Week 3	0.681 \pm 0.168* (15)	0.458 \pm 0.143* (4)	0.626 \pm 0.304* (4)

Means sharing the same letters in a row at any time period are not significantly different ($p < 0.05$).

EGR ACTIVITY vs TIME



Means sharing the same letter at any time period are not significantly different ($p < 0.05$)

Figure 6.7

TABLE 6.8

EGR ACTIVITY (AC) $\bar{X} \pm SD$ (n)

	ENT	PAR2	PAR3
Baseline	1.01 \pm 0.13 ^a (13)	1.06 \pm 0.13 ^a (12)	1.04 \pm 0.15 ^a (13)
Week 1	1.04 \pm 0.07 ^a (15)	1.03 \pm 0.07 ^a (9)	1.00 \pm 0.06 ^a (13)
Week 2	1.02 \pm 0.09 ^{ab} (4)	0.98 \pm 0.07 ^b (6)	1.11 \pm 0.10 ^a (6)
Week 3	0.99 \pm 0.08 ^a (12)	1.00 \pm 0.01 ^a (2)	1.00 \pm 0.01 ^a (6)

Means sharing the same letters in a row at any time period are not significantly different ($p < 0.05$).

URINARY RIBOFLAVIN vs TIME

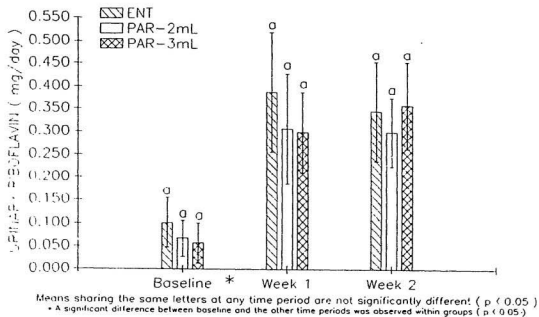


Figure 6.8

TABLE 6.9
URINARY RIBOFLAVIN (mg/d) $\bar{X} \pm SD$ (n)

	ENT	PAR2	PAR3
Baseline*	0.101±0.115* (7)	0.067±0.078* (11)	0.058±0.087* (9)
Week 1	0.387±0.262* (15)	0.306±0.243* (9)	0.299±0.176* (11)
Week 2	0.344±0.215* (13)	0.299±0.151* (5)	0.357±0.188* (3)
Week 3	0.259±0.165 (11)	0.484 (1)	0.197±0.117 (3)

*A significant difference was observed between baseline values and levels at subsequent weeks for all 3 groups ($p < 0.05$).

Means sharing the same letters in a row at any time period are not significantly different ($p < 0.05$).

TABLE 6.10
RIBOFLAVIN BALANCE $\bar{X} \pm SD$ (n)

Group	Intake mg/kg/d	Output mg/kg/d	%Intake Excreted	Net Retention mg/kg/d
ENT				
week 1 (13)	0.368 \pm 0.273	0.307 \pm 0.184	83*	0.055 \pm 0.288
week 2 (13)	0.666 \pm 0.212	0.246 \pm 0.151	37	0.420 \pm 0.224
week 3 (11)	0.654 \pm 0.180	0.173 \pm 0.116	26	0.486 \pm 0.223
PAR2				
week 1 (8)	0.370 \pm 0.205	0.265 \pm 0.192	72	0.078 \pm 0.299
week 2 (5)	0.500 \pm 0.124	0.307 \pm 0.194	61	0.192 \pm 0.204
week 3 (1)	0.490	0.465	-	-
PAR3				
week 1 (11)	0.615 \pm 0.704	0.346 \pm 0.241	56	0.269 \pm 0.680
week 2 (3)	0.713 \pm 0.106	0.408 \pm 0.080	57	0.304 \pm 0.164
week 3 (3)	0.626 \pm 0.304	0.196 \pm 0.105	31	0.431 \pm 0.243

*There is a significant difference between the net retention in weeks 1 and 2 and weeks 1 and 3 for the ENT group ($p < 0.05$).

group were significantly lower than after the initiation of enteral and parenteral feeds. No difference was observed between the 3 groups at any time period. A comparison of intake to output is illustrated in Table 6.10. Mean daily intakes exceeded mean daily excretions resulting in net positive balance. Even so, 39%, 38%, and 31% of the ENT, PAR2, and PAR3 groups respectively were in negative riboflavin balance in the first week. By the second week, 17% of the PAR2 group were still in negative riboflavin balance but the ENT and PAR3 infants were all in positive balance. There was no correlation found between dietary intake and urinary excretion for all the groups except at week 3.

6.5 FOLATE

Data on the tabulated folate intake of infants are presented in figure 6.9 and Table 6.11. The PAR3 infants received significantly more folate than the infants in either the PAR2 or ENT groups in the second week.

A difference was observed in plasma folate between the PAR3 group and both the PAR2 and ENT groups at baseline and week 1 and only between PAR3 and PAR2 infants at week 2 (Figure 6.10, Table 6.13). A time effect was found between week 2 values and those measured at baseline and at week 1 for all study groups. There was no significant difference in RBC folate concentrations between the 3 groups throughout the study or within groups over time (Figure 6.11, Table 6.14).

DIETARY FOLATE VS TIME

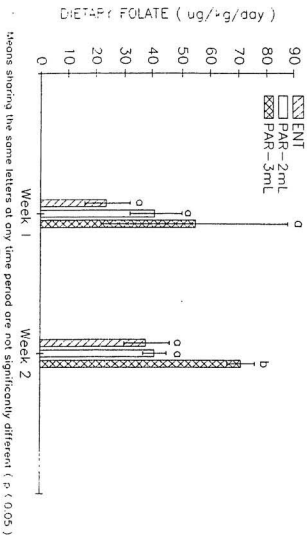


TABLE 6.11
DIETARY FOLATE ($\mu\text{g}/\text{kg}/\text{d}$) $\bar{X} \pm \text{SD}$ (n)

	ENT	PAR2	PAR3
Week 1	24 \pm 15 ^a (15)	41 \pm 18 ^a (12)	55 \pm 65 ^a (13)
Week 2	38 \pm 16 ^a (17)	41 \pm 8 ^a (7)	71 \pm 10 ^b (6)
Week 3	44 \pm 16 ^a (17)	38 \pm 8 ^a (4)	57 \pm 27 ^a (3)

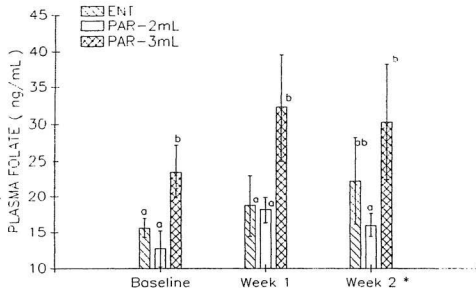
Means sharing the same letters in a row at any time period are not significantly different ($p < 0.05$).

TABLE 6.12

DIETARY FOLATE FROM TPN SOLUTION AT TIME 0 VS DIETARY FOLATE FROM TPN SOLUTION AT TIME 24 FOR THE PARENTERAL GROUPS. $\bar{X} \pm SD$.

	PAR2	PAR3
Time 0		
week 1	41 \pm 18	55 \pm 65
week 2	41 \pm 8	71 \pm 9
week 3	38 \pm 8	57 \pm 27
<hr/>		
Time 24		
week 1	31 \pm 14	41 \pm 48
week 2	31 \pm 6	54 \pm 7
week 3	29 \pm 6	42 \pm 21
<hr/>		

PLASMA FOLATE vs TIME



Means sharing the same letters at any time period are not significantly different ($p < 0.05$)

• A significant difference between week 2 and the other time periods was observed within groups ($p < 0.05$)

Figure 6 10

TABLE 6.13

PLASMA FOLATE (ng/mL) $\bar{X} \pm SD$ (n)

	ENT	PAR2	PAR3
Baseline	16 \pm 3* (9)	13 \pm 5* (10)	24 \pm 7 ^b (9)
Week 1	19 \pm 9* (11)	18 \pm 4* (10)	32 \pm 15 ^b (12)
Week 2*	22 \pm 12 ^{ab} (7)	16 \pm 4* (4)	30 \pm 16 ^b (6)
Week 3	20 \pm 12 (8)	15 (1)	28 \pm 21 (2)

Means sharing the same letters in a row at any time period are not significantly different ($p < 0.05$).

*A significant difference between week 2 values and the values at the other time periods was observed within groups ($p < 0.05$).

RBC FOLATE vs TIME

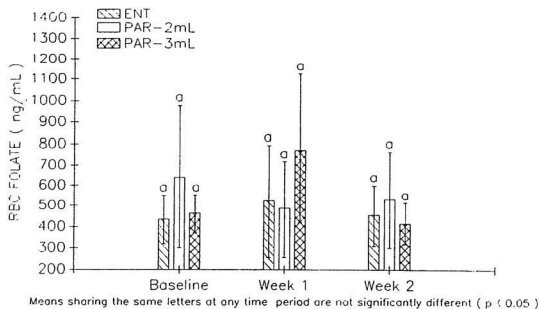


Figure 6.1 I

TABLE 6.14

RBC FOLATE (ng/mL) $\bar{X} \pm SD$ (n)

	ENT	PAR2	PAR3
Baseline	434±231* (13)	639±675* (14)	462±178* (11)
Week 1	525±541* (13)	487±474* (12)	772±705* (15)
Week 2	439±266* (13)	532±463* (5)	416±196* (6)
Week 3	501±359* (9)	947±839* (2)	404±33* (2)

Means sharing the same letters in a row at any time period are not significantly different ($p < 0.05$).

METHOD OF STANDARD ADDITION Urinary Folate (Infants)

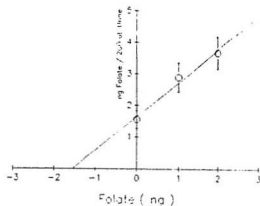


Figure 6.12

METHOD OF STANDARD ADDITION Urinary Folate (Adults)

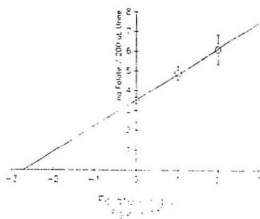


TABLE 6.15

URINARY FOLATE ($\mu\text{g/d}$) $\bar{X} \pm \text{SD}$ (n)

	ENT	PAR2	PAR3
Baseline	1.35 \pm 1.52 (2)	1.03 \pm 0.73 (4)	0.53 (1)
Week 1	2.64 \pm 0.27 (3)	1.56 \pm 0.81 (7)	1.32 \pm 0.54 (3)
Week 2	2.65 \pm 1.67 (5)	1.21 \pm 0.57 (3)	1.37 (1)
Week 3	2.65 \pm 2.04 (2)	1.45 (1)	-

Most infants fell within the normal range for plasma (2-45 ng/mL) (Herbert, 1987a) and RBC folate (>110 ng/mL) concentrations (Becton Dickinson, 1982). Transfused blood samples were analyzed for RBC folate and the mean value was 134 ± 50 ng/mL.

The radioassay procedure used to determine plasma and RBC folate levels could also be used to measure urinary folate concentrations. Figures 6.12 and 6.13 show the results of urine samples from 3 infants and 3 adults using the method of standard additions. Urinary folate output (Table 6.15) appears to follow a similar pattern as seen with thiamin and riboflavin. The baseline levels were lower than levels measured at subsequent weeks. These values are a fraction of the infants' intake and it is evident that all infants are in positive folate balance. (Unfortunately, these samples represented a small proportion of the study group since all but 56 of the urine samples were destroyed due to freezer breakdown).

6.6 VITAMIN B₁₂

Figure 6.14 and Table 6.16 depict dietary vitamin B₁₂ intakes. A significant difference in intakes was observed between the ENT and both parenteral groups in weeks 2 and 3. However, there was no difference in intakes between the 2 parenteral groups. Ideally the PAR2 group was to receive 0.4 µg/d whereas the PAR3 group was to receive 0.6 µg/d but these

DIETARY VITAMIN B12 vs TIME

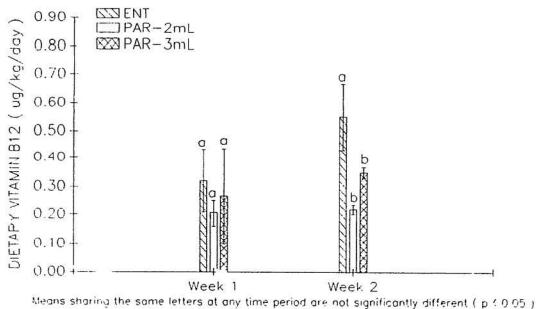


Figure 6.14

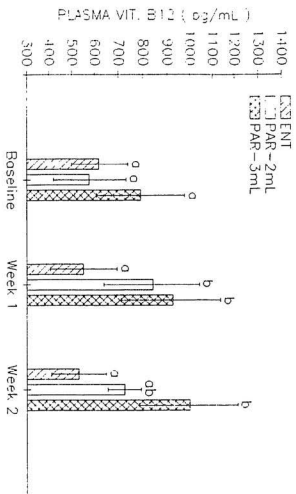
TABLE 6.16

DIETARY VITAMIN B₁₂ ($\mu\text{g/kg/d}$) $\bar{X} \pm \text{SD}$ (n)

	ENT	PAR2	PAR3
Week 1	0.321 \pm 0.225 ^a (15)	0.207 \pm 0.095 ^a (12)	0.268 \pm 0.316 ^a (13)
Week 2	0.548 \pm 0.238 ^a (17)	0.219 \pm 0.038 ^b (7)	0.348 \pm 0.038 ^b (6)
Week 3	0.578 \pm 0.110 ^a (17)	0.238 \pm 0.078 ^b (4)	0.317 \pm 0.194 ^b (3)

Means sharing the same letters in a row at any time period are not significantly different ($p < 0.05$).

PLASMA VITAMIN B12 vs TIME



Means sharing the same letters at any time period are not significantly different ($p < 0.05$)

Figure 6.15

TABLE 6.17

PLASMA VITAMIN B₁₂ (pg/mL) $\bar{X} \pm SD$ (n)

	ENT	PAR2	PAR3
Baseline	618±339 ^a (11)	576±309 ^a (12)	791±368 ^a (11)
Week 1	551±287 ^a (15)	841±405 ^b (13)	924±424 ^b (12)
Week 2	530±238 ^a (11)	725±137 ^{ab} (7)	999±428 ^b (7)
Week 3	631±311 ^a (11)	830±14 ^a (2)	1217±1022 ^a (3)

Means sharing the same letters in a row at any time period are not significantly different ($p < 0.05$).

infants did not receive their allotted amounts every day because of their medical treatment.

There was no significant difference found in plasma vitamin B₁₂ concentrations within groups over time (Figure 6.15, Table 6.17). A difference was observed between the ENT group and both the PAR2 and PAR3 groups at week 1 and between the ENT and PAR3 groups at week 2. Most of the plasma vitamin B₁₂ values were within the normal range of 200-900 pg/mL (Herbert, 1987b). However, 41% of the PAR3 group's vitamin B₁₂ values in the plasma were greater than 900 pg/mL.

Urinary vitamin B₁₂ output could not be detected using the radioassay procedure. Furthermore, urinary methylmalonic acid was not detected in 10 of the 12 samples and only traces were found in the other two samples indicating that there was adequate vitamin B₁₂.

CHAPTER 7.0 DISCUSSION

7.1 INTRODUCTION

The aim of this investigation was to assess the nutritional adequacy of 2 different levels of thiamin, riboflavin, folate and vitamin B₁₂ infusions in LBW infants receiving parenteral nutrition. Large doses of these vitamins may be dangerous to LBW infants due to immature renal function and perhaps impaired urinary excretion, although toxicity from water soluble vitamins is rare (Aldaheff et al, 1984).

In Canada the manufacturer of MVI-Pediatric recommends that 65% of the amount given to full term infants be given to infants weighing less than 3000 grams. This recommendation does not differentiate doses for infants who are significantly less than 3000 g at birth so the question of whether or not 3 mL of this vitamin supplement may be excessive for very low birthweight infants (<1500 g birthweight) requires examination. Interestingly, this is not entirely the situation in the U.S. From the results of Moore's study (1986) and the concern of vitamin E toxicity resulting in neonatal death, the FDA issued the statement that 30% of a vial of MVI-Pediatric should be used for infants weighing less than 1000 g (Greene, 1988). This recommendation has appeared in the package insert of the U.S. manufactured MVI-Pediatric since 1985. Even so, 65% of the vial is still the suggested dose for infants weighing more than 1000 g. The

appropriateness and safety of providing 30% of a vial of MVI-Pediatric to extremely LBW infants has not been assessed for most of the vitamins. However, Phillips and coworkers (1987) did evaluate the adequacy of 30% and 65% of a vial of this multivitamin formulation for infants weighing less than 1000 g for vitamin E status. They found that vitamin E sufficiency was not maintained in all infants receiving 30% of a vial daily. This dose may also have the potential to produce a deficiency of other vitamins. The new guidelines issued by the American Society of Clinical Nutrition in November 1988 are more consistent with using 40% of a unit-dose vial for most vitamins including folate and vitamin B₁₂. More importantly, the recommendations for water soluble vitamins for LBW infants receiving TPN have been revised to coincide more closely with the doses for term infants on a body-weight basis.

7.2 VITAMIN CONCENTRATIONS IN THE TPN ADMIXTURE AND MVI-PEDIATRIC

MVI-Pediatric was mixed with the parenteral solution during the TPN mixture preparation. The infusion of the parenteral solution into the infant usually started 4-6 hours after the end of preparation. The concentration of thiamin, folate, and vitamin B₁₂ were determined in the vitamin solution and in the TPN admixture which was exposed to normal conditions in the Janeway Child Health Centre's Neonatal Unit

during a 30 hour period (Table 6.2).

Unfortunately, the concentration of riboflavin could not be measured in the TPN solution since no assay technique was available for measuring riboflavin 5-phosphate, the form of riboflavin added to MVI-Pediatric. The apoprotein in the fluorometric procedure used in determining urinary riboflavin binds free riboflavin and only loosely binds to the phosphate form (Rhodes et al, 1959). It would be important to measure the riboflavin in the TPN solutions administered at the Janeway Child Health Centre because they are not protected from light. Riboflavin has been found to be inactivated by light especially by the high-intensity light used for phototherapy (Smith et al, 1988). Chen et al (1983) observed that after 8 hours of indirect sunlight, the riboflavin 5-phosphate in a TPN admixture was reduced by 50%. However, despite the high rate of photodegradation expected from TPN solutions normally exposed to light, riboflavin deficiency has not been described in children maintained on TPN. Consequently, due to the unavailability of an acceptable assay, the riboflavin intakes were calculated using data from the product monograph and assuming no degradation over time.

Thiamin instability in TPN has been observed in other studies (Scheiner et al, 1981; Smith et al, 1988). Thiamin losses were attributed to the bisulfite content of the amino acids used and to pH. Significant losses of thiamin were noted at bisulfite concentrations of 3 mEq/L and higher (Smith

et al, 1988). Thiamin levels decreased as bisulfite concentration increased at pH 5.5 but at higher pH, thiamin levels were unchanged. Scheiner et al (1981) observed that the greatest thiamin losses occurred in mixtures of pH 6.5. In the present study, thiamin remained stable over time but 38% more thiamin was detected in the TPN admixture than what was listed in the product monograph of MVI-Pediatric for reasons that are unclear. As well, 35% more thiamin was measured in the multivitamin formulation, itself. Others have not reported similar findings. However, using a similar analytical technique, Smith et al (1988) measured 39% less thiamin than expected at the start of their study (time 0) but did not give an explanation for this discrepancy.

The concentration of folate measured at time 0 in the TPN solution and in MVI-Pediatric was the same as the amount reported to be in the multivitamin preparation. Even so, the concentration of folate decreased significantly by 25% in the TPN admixture over the 30 hour period examined. Smith et al (1988) found no effect of time, temperature, container, amino acid mixture, phototherapy or the presence of Intralipid on the levels of folate. Barker and coworkers (1984) studied the stability of folate in a variety of solutions used for parenteral nutrition. They found that the stability was dependent upon the pH. Maintaining a pH greater than 5 ensured that the folate remained in solution. Most solutions for TPN contain amino acids which are amphoteric and will

exert a buffering capacity ensuring that the pH remains between 5 and 6. These researchers stated that providing the acidity of the solution remains above pH 5, the folate will remain in solution.

Barker et al (1984) also found that folate remained stable in the presence of riboflavin, thiamin, vitamin C, pyridoxine and panthothenyl alcohol in four typical formulations of TPN. However, others (Biamonte et al, 1951; Schniedlin et al, 1952; Tansey et al, 1954) have shown that these vitamins have caused an acceleration in the rate of decomposition of folate which could account for the degradation of folate observed in this investigation.

Due to the expense of the radioassay used in the present study, folate concentration was not measured in the TPN solutions at other time points. It would be of interest to determine the rate of folate degradation over the 24 hour infusion period for a more accurate picture of folate intakes.

There was 33% and 10% less vitamin B₁₂ detected in the TPN admixture and vial of MVI-Pediatric, respectively, than was reported to be present in the vitamin preparation. Using a radioimmunoassay procedure, Marinier et al (1989) also observed 30% less vitamin B₁₂ than expected in the vitamin solution they were studying. Even so, they concluded that they found no substantial loss of any of the vitamins during preparation, storage, or infusion.

The reduction of vitamin B₁₂ detected in the TPN solution

could be due to the presence of ascorbic acid. Vitamin B₁₂ has been shown to degrade in the presence of ascorbic acid. Herbert (1976) observed a destruction of 50-95% of the vitamin B₁₂ content of meals by 0.5 g of vitamin C. He concluded that substantial destruction of crystalline vitamin B₁₂ is brought about by large doses of vitamin C when no other substances are present to protect vitamin B₁₂. This was the reason why earlier multivitamin formulations did not include vitamin B₁₂. Instead, 5 ug of the vitamin was administered intramuscularly on a weekly basis.

7.3 THE BALANCE TECHNIQUE

The concept of "nutritional balance" has been used to determine nutritional status as well as nutrient requirements (Jeejeebhoy, 1986). As part of the assessment of thiamin and riboflavin nutriture, intake was compared to output to determine if the infants were in nutritional balance. However, the balance method could not be used to aid in the interpretation of folate and vitamin B₁₂ for three reasons: (1) there is a slow turnover rate in the body of both these vitamins (Krumdieck et al, 1978; Heyssel et al, 1964), (2) only small amounts are excreted in the urine, and (3) a large majority of folate and vitamin B₁₂ is reabsorbed from the bile.

The concept of nutritional balance is one of a "black box" in which the difference between the input and output is considered to be the balance. If the difference is positive,

it is assumed that the nutrient is accumulating in the body and that the accumulation results in growth and/or functional maintenance and thus, adequate nutritional status. When considering a vitamin, positive balance could indicate that body "stores" are low (e.g., apoenzymes) and thus the patient is vitamin deficient and rapidly attempting to replenish these stores when the vitamin is provided. In contrast, nutrient intakes associated with a negative balance indicate inadequacy (Jeejeebhoy, 1986).

Although the balance method yields valuable information concerning nutrition and metabolism, there are a number of limitations and sources of error with this technique. The fate of the nutrients ingested or infused and their intermediary metabolism are not established from this technique. Furthermore, the intake tends to be overestimated while the output tends to be underestimated. When dealing with TPN solutions there may be losses of the nutrient due to adherence to delivery containers and tubings or due to degradation when exposed to varying environmental conditions which if not accounted for could lead to an overestimation of intake. Unmeasured losses from the skin (including exfoliated cells, sweat, hair and nail growth), flatus, respiration, and blood sampling lead to an underestimation of output (Kopple, 1987). For instance; excess amounts of riboflavin are excreted in the urine but small amounts are also lost in sweat (Cooperman and Lopez, 1984). In the present study, stool

samples were not collected and analyzed for vitamins for the ENT group which could have resulted in a further underestimation of output. It has been observed that 20-30% of ingested thiamin was excreted in the feces of rats (Light et al, 1938). Due to the limitation of balance studies, other parameters of nutritional and metabolic status were used in association with intake and output data to assess the adequacy of the four vitamins examined in this investigation.

7.4 THIAMIN

Ensuring that a LBW infant is receiving appropriate amounts of thiamin is important since thiamin plays a critical role in neurological function (Haas, 1988). Even though toxicity of thiamin is extremely rare, high doses have been associated with respiratory depression and anaphylaxis (Aldaheff et al, 1984). Excess thiamin has been shown to result in an increase in urinary thiamin output (Sauberlich et al, 1974). The relationship of thiamin intake to output in the present study is reported in Table 6.6. The mean net retention was positive for all 3 groups. It is interesting to note that 88%, 50%, and 17% of the thiamin intake was excreted by the ENT, PAR2, and PAR3 groups, respectively, during the first week. In the second week the ENT infants excreted 70% of their intake whereas the infants in the PAR2 group excreted 46% and those in the PAR3 group excreted 40%. The results obtained in the three weeks are extremely

important since the PAR3 group received significantly more thiamin than the other 2 groups. As well, the PAR3 infants who were receiving a parenteral infusion averaging .846 mg thiamin/kg/d were all in positive balance by week 2.

The large urinary thiamin excretion exhibited by infants in the 3 study groups could be attributed to a number of factors. Thiamin excreted in the urine is dependent upon 1) the amount of thiamin ingested, 2) the amount absorbed in the intestine, 3) the metabolism of the individual, 4) the quantity of urine, and 5) the kidney threshold. Benson et al (1942) studied the urinary thiamin excretion of 22 healthy children ranging from 4-10 years of age. These researchers found a variation in thiamin output. The average amount of thiamin excreted was 27% of the amount ingested. They observed that some of the children who received a greater thiamin intake excreted less than others who had a lower intake. They attributed the variation in output to the capacity to absorb the vitamin. The varying degree of absorption was made more apparent when large test doses were given. In fact, Schultz et al (1938) showed that high doses of thiamin were incompletely absorbed.

In the present study, the proportion of thiamin intake excreted dramatically decreased in the second week for the ENT group. Two factors may have influenced this result. Firstly, thiamin is involved in oxidative reactions involving carbohydrates. An increase in carbohydrate calories ingested

and utilized has been shown to result in an increased need for thiamin (Sauberlich, 1964). Since the ENT group was receiving more energy by the second week, this could account for the need to retain more thiamin than previously. Secondly, due to renal immaturity, excretion of thiamin and riboflavin by the infant could be relatively rapid (Jusko et al, 1970). The fact that the subjects' kidneys are not as well developed or functional in the early stages of life and that approximately 25% of parenterally administered vitamins perfuse the kidney before the liver and intestine (Hodges and Dempsey, 1986) could account for the large thiamin output by the PAR2 group during the first week. The reason for the PAR3 group excreting only 17% of their intake in the first week is unclear. The kidney has the ability to favourably adapt to continuous parenteral infusion compared with intermittent enteral ingestion of water soluble vitamins (Schanler, 1988). While the mechanism is still unknown, this could explain why the two parenteral groups excreted less of their intake than the ENT group.

There was adequate thiamin provided according to the TPPE (Figure 6.4, Table 6.4) as was found by others (Moore et al, 1986) for infants receiving 3 mL of MVI-Pediatric. Normal TPPE values fall within the range of 0-15%. Values at the higher percentages indicate that the in vitro addition of TPP was needed for the normal functioning of the enzyme, transketolase. In the ENT group, 36% of the infants at

baseline had TPPE values in the range of 7-10%. These values are normal but could indicate that less thiamin was available for normal functioning at birth. However by week 1, only one child had a TPPE of greater than 5%. In the PAR2 group, 2 infants had TPPE values of 14% and 26% at baseline but these high values were reduced to 6% by week 1 suggesting that once TPN was initiated these infants were receiving adequate thiamin. High values at baseline may imply low stores in utero or low maternal intake. No abnormal TPPE was observed in the PAR3 group.

In thiamin-deficient people transketolase activity is low and can usually be increased by added TPP. However, this increase, or TPP effect, requires the presence of apotransketolase. In some cases, there appears to be no free apoenzyme which can react with TPP, so the transketolase is low but not increased by TPP. Thus Sauberlich (1967) argues that "it appears essential that attention be given to both the TPP stimulation effect and the maximum enzyme activity when transketolase evaluation is considered". In the present study, no difference was found regarding transketolase activity for the 3 groups at weeks 1, 2 or 3 (Table 6.4a) suggesting adequate levels of the apoenzyme.

TPPE does not distinguish between adequate and/or excessive intakes. Therefore whole blood thiamin may serve as a better quantitative measure of thiamin status. The PAR2 group excreted more thiamin than the PAR3 group. (It may be

that blood thiamin concentrations are higher in the PAR3 group than the PAR2 group.

By the second week, the ENT group received a mean intake of 0.259 mg/kg/d, the PAR2 group 0.476 mg/kg/d, and the PAR3 group 0.848 mg/kg/d (Figure 6.3, Table 6.3). In another study (Fritz, 1987 abstract), intravenous intakes of 0.11-0.13 mg/kg/d corrected the erythrocyte transketolase activity for 2 infants deficient in thiamin. Elevated whole blood thiamin levels were seen in term infants who received 0.12 mg/kg/d (Greene et al, 1988). In light of these studies, Greene et al (1988) recommended that LBW infants receiving TPN should receive ~0.35 mg/kg/d of thiamin. Until blood levels are measured, we cannot comment on the appropriateness of this suggestion. In the present study, the PAR2 group received the amount provided by 40% of a vial of MVI-Pediatric. These infants had adequate tissue function and were in positive thiamin balance. Thus, it appears that approximately 0.48 mg/kg/d or less is sufficient for LBW infants receiving TPN.

7.5 RIBOFLAVIN

Phototherapy, the treatment for hyperbilirubinemia, has resulted in riboflavin deficiency in LBW infants receiving enteral feeds (Sisson, 1987; Bates et al, 1985; Lucas and Bates, 1984). Ennever et al (1983a, 1983b) showed that excessive amounts of riboflavin under phototherapy illumination resulted in tissue damage in isolated cells.

Thus, these authors suggested that caution be exercised when providing large supplements of this vitamin to neonates receiving phototherapy. These findings are of particular concern in the present study because 48% of the infants underwent phototherapy in their first week of life.

Since the infants in the parenteral group did not receive all the prepared TPN, the PAR3 infants did not receive significantly more riboflavin than the other two groups (Figure 6.6). When examining riboflavin intake to urinary output in weeks 1 and 2 (Table 6.10), it was observed that 83%, 72%, and 56% of the riboflavin intake was excreted by the ENT, PAR2, and PAR3 infants, respectively, in week 1. Furthermore, 37%, 61%, and 57% of the riboflavin was excreted by the ENT, PAR2, PAR3 infants in the second week. There was a significant difference between the proportion of intake excreted for the ENT group at weeks 1 and 2 ($p < 0.05$).

All 3 groups excreted high percentages of their riboflavin intake, especially in the first week. When given in excess of needs, riboflavin is excreted primarily unchanged in urine. In fact, if body stores are saturated, others have reported quantitative excretion of intake in the urine of adults (Selhub and Rosenberg, 1984). Oldham et al (1942) studied the urinary thiamin and riboflavin excretion in two 5 year old children. They observed an increased riboflavin excretion when the children had upper respiratory infections and no change in thiamin output. The authors stated that this

increase in riboflavin excretion was probably related to a lack of protein deposition and a negative nitrogen balance.

Urinary riboflavin excretion has been shown to be closely related to alterations in nitrogen balance. Sarett et al (1942, 1943) observed that the urinary riboflavin excretion of dogs and rats varied inversely with protein intake and protein deposition. Thus when the protein intake was decreased, the capacity to retain riboflavin was lost. Pollack and Bookman (1951) found that large negative nitrogen balances precipitated by surgical procedures were associated with excretion of riboflavin many times that of the quantity ingested. These authors suggested that any appreciable excess quantity of riboflavin being excreted in the urine could be attributed to the breakdown of flavoproteins. Cayer and Cody (1947) reported a 25% increase in riboflavin excretion in patients with peptic ulcer and suggested that increased excretion might be attributed to body weight loss with resultant riboflavin release from tissues.

At the start of the present study most of the infants had medical problems which may have lead to the large urinary output observed in week 1. By the second week, the ENT group's excretion was lowered by 45% which would be expected since this group improved clinically. However, the riboflavin output only decreased by 19% and 10% for the PAR2 and PAR3 groups, respectively. These infants continued to have medical complications which was exemplified by the fact that they were

still receiving TPN. Nitrogen balance was not assessed in the present study but it should be considered in future investigations to determine if there is a correlation between nitrogen balance and riboflavin excretion in urine for LBW infants.

A functional enzymatic assay was used to determine riboflavin nutriture in this investigation. According to the results, the infants in the ENT, PAR2, and PAR3 groups had adequate riboflavin for normal biochemical functioning (Figure 6.4). Cooperman et al (1973) found that the EGR activity coefficients of cord blood were approximately 1.00 indicating that EGR in neonates at birth was saturated with the riboflavin-containing coenzyme, FAD. Moore et al (1986) had results similar to those found in this investigation for infants weighing less than 2500 g receiving 3 mL of MVI-Pediatric.

EGR activity values greater than 1.20 indicating a riboflavin deficiency, were observed in 1 PAR2 infant and 1 ENT infant at baseline and 1 ENT infant and 1 PAR3 infant at week 2. The high activity coefficients at baseline could be attributed to decreased stores in utero or maternal intake since these were reduced to normal values once feeds were initiated. Surprisingly, the infants with high activity coefficients at baseline were not the same infants with high TPPE's. The 2 infants with high activity coefficients at week 2 did undergo phototherapy in week 1 and the infant in the

PAR3 group received a blood transfusion the day before blood was drawn for the vitamin study. These 2 factors could have contributed to the increase in EGR activity. For the most part, the EGR activity coefficients determined in this study suggest that the infants had adequate riboflavin but they do not indicate whether or not excess riboflavin is being metabolized or if tissue stores are high.

In studying LBW infants, Baeckert and coworkers (1988) observed that a parenteral riboflavin intake of 0.66 mg/kg/d resulted in a 20-400 fold elevation in blood riboflavin levels and an intake of 0.25 mg/kg/d for 2 days resulted in only a 4 fold increase. These authors postulated that this increase reflected an excessive intake and a relatively poor renal clearance of riboflavin. Therefore we suggest that blood concentrations of riboflavin be measured as well as determining functional adequacy and urinary excretion levels.

Fritz (1987, abstract) observed 2 infants who received no riboflavin for 21 days. They both had an EGR activity coefficient indicative of riboflavin deficiency. The abnormal activity coefficients were corrected when riboflavin doses of 0.06 and 0.096 mg/kg/d were given to both patients, respectively, for 2 days. This supports the contention that currently recommended doses of riboflavin are excessive. This finding along with the results from the studies by Baeckert (1988) and Moore (1986) led Greene and coworkers (1988) to suggest that 0.15 mg/kg/d of riboflavin would be adequate for

LBW infants receiving TPN. As intakes of these low levels were not evaluated, comment on the appropriateness of these recommendations is not possible. However, from the results of this investigation, we can suggest that approximately 0.45 mg/kg/d of riboflavin or less is adequate for LBW infants being parenterally fed.

7.6 FOLATE

Since folate is required for growth and erythropoiesis (Rodriguez, 1980) LBW infants may have a need for higher supplementation than term infants. There is a scarcity of data available on the effects of excess folate. However, Ford et al (1975) suggested that excess folate may have an adverse affect on the microbial population in the intestine of infants by increasing the growth of folate-requiring bacteria. Consequently, it may not only be a matter of folate supplementation but a matter of therapeutically appropriate dosages which require consideration or investigation.

Little information is available about folate levels in plasma and red blood cells during and after long term supplementation with folate in TPN. Determining RBC folate concentration is the most common index used to assess folate status. From the results (Table 6.14), the infants in all 3 groups had adequate folate stores. Infants in the present study had plasma folate concentrations of 19 ± 9 ng/mL, $18 \pm$

4 ng/mL, and 32 ± 15 ng/mL for the ENT, PAR2, and PAR3 groups, respectively, in the first week and 22 ± 12 ng/mL, 16 ± 4 ng/mL, and 30 ± 16 ng/mL in the second (Figure 6.10). The PAR3 infants received significantly more folate than the other 2 groups in week 2. Surprisingly, the PAR3 group's mean plasma folate level did differ from that of the ENT and PAR2 groups prior to the initiation of feeds and at week 1 but differed only from the PAR2 group at week 2.

Two infants in the ENT group and 2 in the PAR3 group had high plasma folate values during the course of the study. One of the infants in the ENT group was switched to TPN by the second week due to medical complications that could account for the higher value. The other infant had many problems at birth including sepsis, hypoglycemia and jaundice. Furthermore, this child had high plasma vitamin B₁₂ concentrations throughout the study. The 2 infants in the PAR3 group with elevated plasma folate levels also had high plasma vitamin B₁₂. Both of these infants had multiple medical problems, high concentrations of RBC folate and blood transfusions by week 2. It may be possible that all four infants had difficulty with the metabolic handling of these vitamins.

There was no correlation found between dietary folate and plasma folate for all three groups at any time period. As well, a comparison of intake to output was tabulated but, unfortunately, there were too few samples, especially by the

second week, to be able to determine statistical significance. Only a small proportion of the intake was excreted in urine, indicating that most of the folate is probably reabsorbed.

Tamura et al (1980) studied term infants (3-25 weeks) receiving breast milk which contained a mean folate concentration of 141 ng/mL. Their daily intake ranged from 14-25 ug/kg/d. Plasma folate and RBC folate levels were 29 ± 14 ng/mL and 429 ± 186 ng/mL, respectively. These values are similar to those of Smith et al (1985) who studied infants from birth to 6 months. Ek and Magnus (1979) analyzed plasma and RBC folate in breast fed infants during their first year of life. Plasma folate values were 13-20 ng/mL and RBC folate values were 264-396 ng/mL. The results from these studies appear to be consistent with the folate concentrations determined in the present investigation. More importantly, Moore et al (1986) measured RBC folate in LBW infants receiving TPN and their results were similar to those observed in this study.

Ek (1988) suggested that 65 ug/d or ~15 ug/kg/d would be adequate for LBW infants whereas Greene et al (1988) recommended 56 ug/kg/d for those infants receiving parenteral feeds. By the second week the PAR2 group was more like the control group (ENT) than the PAR3 group in the amount of folate received and the folate concentration measured in plasma. Therefore, it appears that 40 ug/kg/d, the amount received intravenously by the PAR2 infants, would be

sufficient for LBW infants receiving TPN.

7.7 VITAMIN B₁₂

Vitamin B₁₂ has an important role in folate metabolism for 2 reasons; 1) it is a constituent of methylcobalamin, a cofactor necessary for folate metabolism, and 2) it is involved in the regeneration of folate for nucleic acid metabolism (Ellenbogen, 1984; Herbert, 1987b; Shinton, 1972). These two factors suggest that vitamin B₁₂ is essential for the developing infant.

Vitamin B₁₂ as well as folate and vitamin K can be synthesized by bacteria in the gut. However, antibiotics were administered to 79% of the infants in this study as part of their medical treatment and more than likely, interfered with the microbial production of these vitamins. Therefore, it is imperative that these infants receive vitamin B₁₂ either intravenously or intramuscularly.

Since there is no difference between the amount of vitamin B₁₂ the PAR2 and PAR3 infants received, the results of both groups can only be compared to the data obtained for the ENT group. The ENT group received significantly more vitamin B₁₂ than the PAR2 and PAR3 groups in week 2. Surprisingly, the PAR3 group had significantly higher plasma vitamin B₁₂ values than the ENT infants in weeks 1 and 2. In fact, 41% of the samples from the PAR3 group had levels greater than 900 pg/mL whereas only 15% of the PAR2 and 8% of the ENT samples were

above this value. Moore et al (1986) obtained plasma vitamin B₁₂ levels in the range of 1000-2900 pg/mL in their group of 18 LBW infants receiving 3 mL of MVI-Pediatric. These researchers observed no significant difference over time which is consistent with the results of this study. Furthermore, there was no correlation found between dietary vitamin B₁₂ and plasma vitamin B₁₂ for all three groups at any time period.

No vitamin B₁₂ was detected in the urine that was analyzed and only traces of urinary methylmalonic acid were measured suggesting no deficiency. It is true that a large percentage of vitamin B₁₂ is absorbed; however, it has been observed that following intravenous administration 50-90% of the dose is excreted in the urine within 48 hours in adults (Ellenbogen, 1984). Either all the vitamin B₁₂ is reabsorbed due to increased need or the negligible output is the result of poor renal clearance leading to the high blood levels seen in the PAR3 infants.

Since a marked elevation of plasma vitamin B₁₂ was observed with infants receiving 0.6 ug vitamin B₁₂/kg/d intravenously (Moore et al, 1986), Greene and coworkers (1988) suggested that 0.4 ug/kg/d would be adequate for LBW infants being parenterally fed. This amount is consistent with 40% of the amount of vitamin B₁₂ recommended for full term infants receiving TPN and thus, the amount given to infants receiving 2 mL of MVI-Pediatric. Because the majority of plasma vitamin B₁₂ values fell within the normal range of 200-900 pg/mL for

the PAR2 group and the fact that the mean plasma values did not differ from that of the control group, it appears that 0.20-0.25 ug/kg/d is sufficient for LBW infants receiving TPN.

Chapter 8.0 CONCLUSION

From the results of this study, it appears that supplying intravenously fed LBW infants 1.5-1.6 mL of MVI-Pediatric (the actual mean amount given to the PAR2 infants [Table 6.1a]) is sufficient to maintain normal biochemical functioning of thiamin, riboflavin, folate and vitamin B₁₂. However, if the infants had received their full complement of the vitamin formulation, the amount of riboflavin, folate, and vitamin B₁₂ may have then been excessive. We can further suggest that providing approximately 0.48 mg/kg/d of thiamin, 0.45 mg/kg/d of riboflavin, 40 µg/kg/d of folate and 0.20 - 0.25 µg/kg/d of vitamin B₁₂ would be adequate for LBW infants receiving TPN.

LBW infants are difficult to study because of their vulnerability and need for constant intensive care. Even so, the need to determine their nutrient requirements is imperative. This investigation has provided the framework for future research to be carried out with this population group. The outcome of this study along with the many problems experienced with the experimental design has led to insight and suggestions that will benefit further investigative work in this area:

- 1) Infants may not receive their total TPN infusion. This is an important observation for future balance studies. If possible, these infants should receive the full complement of the nutrient being examined.

- 2) Developing a statistical method for dealing with missing values is essential for this kind of study that precludes the use of the repeated measures of analysis design.
- 3) Blood and fecal levels as well as functional enzymatic assays and urinary excretion should be determined for a more definitive interpretation of thiamin and riboflavin nutriture.
- 4) Using a dose response curve design would allow for a more accurate determination of vitamin requirements.
- 5) Actual vitamin concentrations in TPN solutions and the vitamin formulation should be measured to determine the accuracy of the product monograph and to observe possible degradation over the entire infusion period.
- 6) The unit dose of any of the vitamins should be recommended on a body weight basis which is consistent with the current trend for other nutrient requirements for LBW infants.

REFERENCES

- Aldaheff L, Gualtieri T, Lipton M. 1984. Toxic effects of water soluble vitamins. *Nutr Rev* 42(2):33-40.
- American Academy of Pediatrics/Committee on Nutrition, 1976. Commentary on breast feeding and infant formulas, including proposed standards for formulas. *Pediatr* 57:2178-285.
- American Medical Association/Nutrition Advisory Group, 1979. Multivitamin preparation for parenteral use. A statement by the nutrition advisory group, 1975. *JPEN* 3(4):258.
- American Academy of Pediatrics Committee on Nutrition. 1967. Proposed changes in food and drug administration regulations concerning formula products and vitamin-mineral dietary supplements for infants. *Pediatr* 40:916-922.
- American Academy of Pediatrics/Committee on Nutrition, 1977. Nutritional needs of low birthweight infants. *Pediatr* 60(4):519.
- American Academy of Pediatrics/Committee on Nutrition, 1985. Nutritional needs of low birthweight infants. *Pediatr* 75:976.
- Anderson GH. 1985. Human milk feeding. *Pediatr Clin N Am* 32(2):335-353.
- Anderson TL et al. 1979. A controlled trial of glucose vs glucose and amino acids in premature infants. *J Pediatr* 94:947.
- Asfour R, Wahbeh N, Waslien CI, et al. 1977. Folic acid requirement of children. III. Normal infants. *Am J Clin Nutr* 30:1098-1105.
- Atkinson et al, 1980. Macro-mineral content of milk obtained during early lactation from mothers of premature infants. *Early Hum Dev* 4:5.
- Atkinson SA, Bryan MH, Anderson GH. 1978. Human milk: Difference in nitrogen concentration in milk from mothers of term and premature infants. *J Pediatr* 93:67.
- Atkinson SA, Bryan MH, Anderson GH. 1981. Human milk feeding in premature infants: Protein, fat and carbohydrate balances in the first two weeks of life. *J Pediatr* 99:617.

- Baekert PA, Greene HL, Fritz I, et al. 1988. Vitamin concentrations in very low birthweight infants given vitamins intravenously in a lipid emulsion: Measurement of vitamins A, D, and E and riboflavin. *J Pediatr* 113:1057-1065.
- Barker A, Hebron BS, Beck PR et al. 1984. Folic acid and total parenteral nutrition. *JPEN* 8(1):3-13.
- Bates CJ, Liu DS, Fuller NJ, Lucas A. 1985. Susceptibility of riboflavin and vitamin A in breast milk to photodegradation and its implications for the use of banked milk in infant feeding. *Acta Pediatr Scand* 74:40-44.
- Beatty RD. Concepts, instrumentation and techniques in atomic absorption spectrophotometry. Perkin Elmer Corporation, 1978.
- Benn A, Swan C, Cooke WT et al. 1971. Effect of intraluminal pH on the absorption of pteroylmonoglutamic acid. *Br Med Jr* 1, 148-150.
- Benson RA, Witzberger CM, Slobody LB. 1941. The urinary excretion of thiamin in normal children. *J Pediatr* 18:617-620.
- Biamonte AR, Schneller GH. 1951. A study of folic acid stability in solutions of the B complex vitamins. *J Am Pharmacol Assoc (Sci ed)* 40:313-320.
- Brady T, Shane B, Stoksted ELK. Folic acid. In: Handbook of vitamins-nutritional, biochemical, and clinical aspects. Ed: Machlin LJ. New York: Marcel Dekker Inc., 1984.
- Brin M, Dibble MV, Peel A, et al. 1965. Some preliminary findings on the nutritional status of the aged in Onondaga County, N.Y. *Am J Clin Nutr* 17:240-258.
- Brooke OG. 1987. Nutritional requirements of low and very low birthweight infants. *Ann Rev Nutr* 7:91-116.
- Burch HB, Bessey OA, Lowry OH. 1948. Fluorometric measurements of riboflavin and its natural derivatives in small quantities of blood, serum and cells. *J Biol Chem* 175:457.
- Burland WL, Simpson K, Lord J. 1971. Response of low birthweight infants to treatment with folic acid. *Arch Dis Child* 46:189-194.

- Cayer D and Cody S. 1947. The nutritional state of patients with peptic ulcer as measured by vitamin levels in the blood and urine. *Gastroenterology* 9:54-63.
- Challacombe D. 1974. Bacterial microflora in infants receiving nasojunal tube feeding. *J Pediatr* 85:113.
- Chen MF, Boyce HW, Triplett L. 1983. Stability of the B vitamins in mixed parenteral nutrition solution. *JPEN* 462-464.
- Chessex P and Zebich H. 1985. Effects of amino acid composition of parenteral solutions on nutrition retention and metabolic responses in low birthweight infants. *J Pediatr* 106:1111.
- Collins JE, Rolles CJ, Sutton H et al. 1984. Vitamin B₁₂ absorption after necrotizing enterocolitis. *Arch Dis Child* 59(8):731-734.
- Cooper BA and Rosenblatt DS. 1987. Inherited defects of vitamin B₁₂ metabolism. *Ann Rev Nutr* 7:291-320.
- Cooperman JM, Pesci-Bourel A, Lohby AL. 1970. Urinary excretion of folic acid activity in man. *Clin Chem* 16:375-381.
- Cooperman JM and Lopez R. 1984. Riboflavin. In: *Handbook of vitamins*. Ed: Machlin LJ. New York: Marcel Dekker Inc., 1984.
- Cooperman JM, Cole HS, Gordon M, et al. 1973. Erythrocyte glutathione reductase as a measure of riboflavin nutritional status of pregnant women and newborns. *Proc Soc Exp Bioc Med* 143:326.
- Corcino JJ, Coll G, Klipstein FA. 1975. Pteroylglutamic acid malabsorption in tropical sprue. *Blood* 45:577-580.
- Cowett RM, Oh W, Pollak A et al. 1979. Glucose disposal of low birth weight infants: steady state hyperglycemia produced by constant intravenous glucose infusion. *Pediatr* 63(3):389-396.
- Cremer RJ, Perryman PW, Richards DH. 1958. Influence of light on the hyperbilirubinemia of infants. *Lancet* 1:1094.
- Dallman PR. Nutritional anemia of infancy: iron, folic acid, and vitamin B₁₂. In: *Nutrition during infancy*. Ed: Tsang RC, Nichols BL. Toronto: Hanley and Belfus Inc., 1988.

- Davies DP. 1977. Adequacy of expressed breastmilk for early growth of preterm infants. *Arch Dis Child* 52:296.
- Dencker H, Jagerstad M, Westesson AK. 1976. Total and peripheral plasma folates after ingestion of folic acid. *Acta Hepatogastroenterol* 23:140-143.
- Denko CW, Grandy WE, Porter JW. 1946. The excretion of B-complex vitamins in the urine and feces of seven normal adults. *Arch Biochem* 10:33-40.
- Dudrick SJ. 1968. Long-term TPN with growth, development and positive nitrogen balance. *Surgery* 64:134.
- Duffy B, Gunn R, Collenge J, Pencharz P. 1981. The effect of varying protein quality and energy intake on the nitrogen metabolism of parenterally fed LBW infants. *Pediatr Res* 15:1040.
- Dweck HS and Cassady G. 1979. The role of hepatic control of glucose homeostasis in the etiology of neonatal hypo and hyperglycemia. *Seminars in Perinatology*. 3:327-340.
- Ek J and Magnus EM. 1979. Plasma and red blood cell folate in breastfed infants. *Acta Paediatr Scand* 68:239.
- Ek J and Magnus E. 1980. Plasma and red cell folacin in cow's milk-fed infants and children during the first 2 years of life: the significance of boiling pasteurized cow's milk. *Am J Clin Nutr* 33:1220-1224.
- Ek J and Magnus E. 1982. Plasma and red cell folate values and folate requirements in formula-fed infants. *J Pediatr* 100(5):739-744.
- Ek J. Folic acid and vitamin B12 requirements in premature infants. In: *Vitamin and mineral requirements in preterm infants*. Ed: Tsang RC. New York: Dekker Inc, 1988.
- Ellenbogen L. Vitamin B₁₂. In: *Handbook of vitamins-nutritional, biochemical, and clinical aspects*. Ed: Machlin LJ. New York: Marcel Dekker Inc., 1984.
- Ennever JT and Speck WT. 1983a. Photochemical reactions of riboflavin: covalent binding to DNA and to Poly(dA).Poly(dT). *Pediatr Res* 17:234-236.
- Ennever JT, Carr HS, Spect WT. 1983b. Potential for genetic damage from multivitamin solutions exposed to phototherapy illumination. *Pediatr Res* 17:192-194.

- Fitzharding et al. 1976. Mechanical ventilation of infants less than 1500 gram birth weight; health, growth, and neurological sequelae. *J Pediatr* 88:531-554.
- Fomon SJ, Ziegler EE, Vazquez HD. 1977. Human milk and the small premature infant. *Am J Dis Child* 131:463.
- Fomon SJ. Infant Nutrition. Philadelphia: WB Saunders and Co., 1974.
- Ford JE, Zechalko A, Murphy J et al. 1983. Comparison of the B vitamin composition of milk from mothers of preterm and term babies. *Arch Dis Child* 58:367.
- Ford JE and Scott KJ. 1975. Some observations on the possible nutritional significance of vitamin B₁₂-and folate-binding proteins in milk. Absorption of [⁵⁷Co] cyanocobalamin by suckling piglets. *Br J Nutr* 34:469-492.
- Fritz I, Said H, Harris C et al. 1987, abstract. A new sensitive assay for plasma riboflavin using high performance liquid chromatography. *J Am Coll Nutr* 6:449.
- Gandy GM, Adamsons K, Cunningham N, Silverman WA, James LS. 1964. Thermal environment and acid-base homeostasis in human infants during the first few hours of life. *J Clin Invest* 43:751-758.
- Gandy G and Jacobson W. 1977. Influence of folic acid on birthweight and growth of the erythroblastic infant. III. Effect of folic acid supplementation. *Arch Dis Child* 52:16.
- Gerson CD, Cohen N, Hepner GW et al. 1971. Folic acid in man: Enhancing effect of glucose. *Gastroenterology* 61:224-227.
- Gluber CJ. Thiamin. In: Handbook of vitamins - nutritional, biochemical and clinical aspects. Ed: Machlin LJ. New York: Marcel Dekker Inc., 1984.
- Green HL, Hambidge KM, Schanler R, et al. 1988. Guidelines for the use of vitamins, trace elements, calcium, magnesium, and phosphorus in infants and children receiving total parenteral nutrition: report of the Subcommittee on Pediatric Nutrient Requirements for the Committee on Clinical Practice Issues of the American Society for Clinical Nutrition. *Am J Clin Nutr* 48:1324-1342.
- Greene HL. 1988 (letter). Vitamin dosages for premature infants. *Pediatr* 81:173.

- Greene HL. Disorders of water soluble vitamins, B-complex and vitamin C. In: Textbook of pediatric nutrition. Ed: Suskind RM. New York: Raven Press, 1981.
- Gross SJ, David RJ, Bauman L, Tamarelli RM. 1980. Nutritional composition of milk produced by mothers delivering preterm. J Pediatr 96(4):641-644.
- Gross SJ et al. 1981. Elevated IgA concentration in milk produced by mothers delivered of preterm infants. J Pediatr 99:389.
- Haas RH. 1988. Thiamin and the brain. Ann Rev Nutr 8:483-515.
- Halstead CH, 1979. The intestinal absorption of folates. Am J Clin Nutr 32:846-855.
- Handbook of Vitamins - nutritional, biochemical and clinical aspects. Ed Machlin New York: Marcel Dekker Inc., 1984.
- Handbook of Neonatal Intensive Care. Ed: Halliday HL, McClure G, Reid M. East Sussex: Bailliere Tindall, 1985.
- Heird WL. 1974. Nasojejunal feeding: a commentary. J Pediatr 85:111.
- Heird WC and Winters RW. 1975. Total parenteral nutrition: the state of the art. J Pediatr 86:2.
- Henshaw JL, Noakes G, Morris SO. 1970. Method for evaluating thiamine adequacy in college women. J Am Diet Assoc 57:436.
- Herbert V, Jacob E, Wong KTJ. 1977. Destruction of vitamin B₁₂ by vitamin C. Am J Clin Nutr 30:297-303.
- Herbert V. Nutritional anemias of childhood- folate, B₁₂: The megaloblastic anemias. In: Textbook of pediatric nutrition. Ed: Suskind RM. New York: Raven Press, 1983.
- Herbert V. 1987b. Recommended dietary intakes (RDI) of vitamin B₁₂ in humans. Am J Clin Nutr 45:671-678.
- Herbert V. 1987a. Recommended dietary intakes (RDI) of folate in humans. Am J Clin Nutr 45:661-670.
- Heyssel RM, Bozian RL, Darby WJ, et al. 1966. Vitamin B₁₂ turnover in man. The assimilation of vitamin B₁₂ from natural foodstuffs by man and estimates of minimal dietary requirements. Am J Clin Nutr 18:176-184.

- Higginbottom MC, Sweetman L, Nyhan WL. 1978. A syndrome of methylmalonic aciduria, homocystinuria, megaloblastic anemia and neurologic abnormalities in a vitamin B₁₂-deficient breastfed infant of a strict vegetarian. *N Eng J Med* 299(7):317-323.
- Hodges RE and Dempsey D. Parenteral vitamin therapy in hospital patients. In: *Parenteral Nutrition* (volume 2). Ed: Rombeau JL and Caldwell MD. Toronto: WB Saunders Co, 1986.
- Hoffbrand AV, Newcombe BFA, Mollin DL. 1966. Method of assay of red cell folate activity and the value of the assay as a test of folate deficiency. *J Clin Pathol* 19:17-28.
- Hommes OR and Obbens EA. 1972. The epileptogenic action of Na-folate in the rat. *J Neurol Sci* 16:271-281.
- Hovi L, Hekali RL, Simes MA. 1979. Evidence of riboflavin depletion in breast-fed newborns and its further acceleration during treatment of hyperbilirubinemia by phototherapy. *Acta Paediatr Scand* 68:567-570.
- Hsu JM, Kawin B, Minor P, et al. 1966. Vitamin B₁₂ concentrations in human tissues. *Nature* 210:264-265.
- Jadhav M, Webb JKG, Vaishnava S, et al. 1962. Vitamin B₁₂ deficiency in Indian infants; a clinical syndrome. *Lancet* ii:903-907.
- James BE, Hendry PS, MacMahon RA. 1975. TPN of premature infants. 2. Requirement for micronutrient elements. *Aust Paediatr J* 15:67-71.
- Jeejeebhoy KN. 1986. Nutritional balance studies: indicators of human requirements or adaptive mechanisms. *J Nutr* 116:2061-2063.
- Johnson PR and Roloff JS. 1982. Vitamin B₁₂ deficiency in an infant strictly breast-fed by a mother with latent pernicious anemia. *J Paediatr* 100:917-919.
- Joshi A, Gerhardt T, Shandloff P, Bancalari E. 1987. Blood transfusion effect on the respiratory pattern of preterm infants. *Paediatr* 80(1):79-84.
- Jusko WJ, Khanna M, Levy G et al. 1970. Riboflavin absorption and excretion in the neonate. *Paediatr* 45(6):945.
- Jusko WT and Levy G. 1975. Riboflavin. Ed. Rivkin RS. New York: Plenum Press.

- Keagy PM, Folates: Microbiological and animal assays In: Methods of vitamin Assay Ed: Augustyn J, Klein BP, Becker D, Venugopal PB. Toronto: John Wiley & Sons, 1985.
- Kende G, Ramot B, Grossowicz N. 1963. Blood folic acid and vitamin B₁₂ activities in healthy infants and infants with nutritional anemias. Br J Haematol 9:328-335.
- Kerner JA. Water soluble vitamins. In: Manual of pediatric parenteral nutrition. New York: John Wiley and sons, 1983.
- Khalil M, Talios A, Moghazy M, et al. 1973. Serum and red cell folates and serum vitamin B₁₂ in protein calorie malnutrition. Arch Dis Child 48:366-369.
- Komrower GM and Robins AJ. 1969. Plasma amino acid disturbance in infancy. I. Hypermethioninaemia and tyrosinemia. Arch Dis Child 44:416-421.
- Kopple JD. 1986. Uses and limitations of the balance technique. JPEN 1(5):795-855.
- Krumdieck CL, Fukushima K, Fukushima T et al. 1978. A long-term study of the excretion of folate and pterins in a human subject after ingestion of ¹⁴C folic acid, with observations on the effect of diphenylhydantoin administration. Am J Clin Nutr 31:88-93.
- Landwith J. 1972. Continuous nasogastric infusion versus total intravenous alimentation. J Pediatr 81:1037.
- Lemons JA, Moyi L, Hall D, Simmons M. 1982. Differences in the composition of preterm and term human milk during early lactation. Pediatr Res 16:113.
- Leveille G. 1972. Modified thiochrome procedure for the determination of urinary thiamin. Am J Clin Nutr 25:273-275.
- Levin DL, Stanger P, Herman JA, Heyman MA. 1971. Congenital heart disease in LBW infants. Circulation 52:500-503.
- Light RF, Schultz AS, Atkin L et al. 1938. Excretion of vitamin B₁ in urine and feces. J Nutr 16:333-341.
- Lucas A and Bates C. 1984. Transient riboflavin depletion in preterm infants. Arch Dis Child 59:837-841.
- Lucey JF. 1972. Neonatal jaundice and phototherapy. Pediatr Clin of N Am 19:827-839.

- Luhby AL. 1959. Megaloblastic anemia in infancy. III Clinical considerations and analysis. *J Pediatr* 54:617-632.
- Macver JE and Back EH. 1960. Megaloblastic anemia of infancy in Jamaica. *Arch Dis Child* 35:134-145.
- Manser JE and Brodsky ML. 1985, abstract. Serum B₁₂ and folate levels in low birthweight infants: a reassessment of recommended intravenous supplements. *Pediatr Res* 351A.
- Marinier E, Gorski AM, Potier De Courcy. 1989. Blood levels of water-soluble vitamins in pediatric patients on total parenteral nutrition using a multiple vitamin preparation. *JPEN* 13(2):176-184.
- Marks J. Vitamin B₁. In: The vitamins, their role in medical practise. Lancaster: MTP Press Ltd., 1985.
- Maslenikova EM. 1963. Effect of light on riboflavin metabolism. *Fed Proc Transl Suppl* 22:T605-T607.
- Matoth Y, Zehavi I, Topper E, et al. 1979. Folate nutrition and growth in infancy. *Arch Dis Child* 54:699-702.
- Matoth Y, Pinkas A, Sroka C, et al. 1965. Studies on folic acid in infancy. III. Foliates in breast fed infants and their mothers. *Am J Clin Nutr* 16:356-359.
- McCoy EE, Strynadka K, Schiff D. 1985, abstract. Serial data blood B₆ vitamin levels in breast, formula, and parenterally fed premature infants. *Pediatr Res* 227A.
- McLaren DS. 1981. The luxur vitamins- A and B₁₂. *Am J Clin Nutr* 34:1611-1616.
- Meisch P, Jahnig D, Jahnig K. 1978. Photodegradation of bilirubin as enhanced by photosensitizers in vitro. *Acta Biol Med Ger* 37(8):1229-1235.
- Meloni T, Corti R, Naitana AF et al. 1982. Lack of effect of phototherapy on red cell riboflavin status and on glucose-6-phosphate dehydrogenase activity in normal and G-6-PD-deficient subjects with neonatal jaundice. *J Pediatr* 100(6):972-974.
- Mendelson RA, Bryan MH, Anderson GH. 1983. Trace mineral balance in preterm infants fed their own mother's milk. *J Pediatr Gastroenterol Nutr* 2:256.
- Methods of Vitamin Assay. Ed: Augustyn J, Klein BP, Becker

- D, Venugopal PB. Toronto: John Wiley & Sons, 1985.
- Mock DM. Water soluble vitamins-supplementation and the importance of biotin. In: Total parenteral nutrition. Ed: Lebenthal E. New York: Raven Press, 1986.
- Moore MC, Greene ML, Phillips B. 1986. Evaluation of a pediatric multivitamin preparation for total parenteral nutrition in infants and children. I. Blood levels of water soluble vitamins. *Pediatr* 77(4):530-538.
- Morley CJ. The respiratory distress syndrome. In: Textbook of neonatology. Ed: Robertson NRC. New York: Churchill Livingstone, 1986.
- Oldham H, Johnston F, Kleiger S et al. 1944. A study of the riboflavin and thiamin requirements of children of preschool age. *J Nutr* 27:435-446.
- Organic Acids in Man. Ed: Chalmers RA and Lawson AM. New York: Chapman and Howe, 1982.
- Orzalesi M. 1987. Vitamins and the premature. *Biol Neonate*.
- Orzalesi M and Colarizi P. 1982. Critical vitamins for low birthweight infants. *Acta Pediatr Scand Suppl* 296:104.
- Parker P, Stoop S, Greene H. 1981. A controlled comparison of continuous versus intermittent feeding in the treatment of infants with intestinal disease. *J Pediatr* 99:360.
- Pereira GR and Lemons JA. 1981. Controlled study of transpyloric and intermittent gavage feeding in the small preterm infant. *Pediatrics* 67:68.
- Pereira GR and Glassman, M. Parenteral nutrition in the neonate. In: Textbook of pediatric nutrition. Ed: Suskind RM. New York: Raven Press, 1983.
- Pereira GR and Barbosa NMM. 1986. Controversies in neonatal nutrition. *Pediatr Clin N Am* 33(1):65-89.
- Pereira GR et al. 1981. Hyperalimentation-induced cholestasis. *Am J Dis Child* 135:842.
- Phillips B, Franck LS, Greene HL. 1987. Vitamin E levels in premature infants during and after intravenous multivitamin supplementation. *Pediatr*. 80(5):680-683.
- Pitcher-Wilmott R, Shutack JG, Fox WW. 1979. Decreased lung volume after nasogastric feeding of neonates recovering

- from respiratory disease. *J Pediatr* 95:119-121.
- Pollack H and Poolman JJ. 1951. Riboflavin excretion as a function of protein metabolism in the normal, catabolic and diabetic human being. *J Lab Clin Med* 38:561-573.
- Ramasatri BV. 1965. Folate activity in human milk. *Br J Nutr* 19:581-586.
- Recommended Nutrient Intakes for Canadians. Ed: Hawkins WW. Bureau of Nutritional Sciences Ottawa: Health and Welfare Canada, 1983.
- Reichman BL, Chessex P, Putet G. 1982. Partition of energy metabolism and energy cost of growth in the very low birthweight infant. *Pediatrics* 69:446.
- Rhea JW and Kilby JO. 1970. A nasojejunal tube for infant feeding. *Pediatr* 46:36.
- Rhodes MB, Bennett N, Feeney RE. 1959. The flavoprotein-apoprotein system of egg white. *J Bioc Chem* 234(8):2054-2060.
- Rindi G and Ventura U. 1972. Thiamin intestinal transport in humans. *Physiol Rev* 52:821-827.
- Ritland S, Fausa O, Gjone E, et al. 1975. Effect of treatment with a bile-sequestering agent (Secholex) on intestinal absorption, duodenal bile acids, and plasma lipids. *Scand J Gastroenterol* 10:791-800.
- Rivlin RS. 1970a. Riboflavin metabolism. *N Engl J Med* 283:463.
- Rivlin RS. 1970b. Regulation of flavoprotein enzymes in hypothyroidism and in riboflavin deficiency. *Adv Enzyme Regul* 8:239-250.
- Rodriquez MS. Folic acid requirements of man. In: Nutritional requirements of man: A conspectus of research. Ed: Irwin MI. New York: Nutrition Foundation, Inc., 1980.
- Roe DA, McCormick DB, Lin RT. 1972. Effects of riboflavin on boric acid toxicity. *J Pharm Sci* 61:1081-1085.
- Ronholm KAR. 1986. Need for riboflavin supplementation in small prematures fed with human milk. *Am J Clin Nutr* 43:1-6.
- Rowe RD, Freedom RM, Mehrizi A, Bloom KR. Patent Ductus Arteriosus. In: The neonate with congenital heart

- disease. Philadelphia: WB Saunders, 1981.
- Roy RN, Pollnitz RP, Hamilton JR et al. 1977. Impaired assimilation of nasojejunal feeds in healthy newborn infants. *J Pediatr* 90:431-434.
- Salmenpera L, Perheentupa J, Siimes MA. 1986. Folate nutrition is optimal in exclusively breastfed infants but inadequate in some of their mothers and in formula-fed infants. *J Pediatr Gastroenterol Nutr* 5(2):284-288.
- Sarasohn C. 1977. Care of the very small premature infant. *Pediatr Clin N Am* 24(3):619-632.
- Sarett HP, Klein JR, Perlzweig WA. 1942. The effect of the level of protein intake upon the urinary excretion of riboflavin and nicotinic acid in dogs and rats. *J Nutr* 24:295-306.
- Sarett HP and Perlzweig WA. 1943. The effect of protein and B-vitamin level of the diets upon the tissue content and balance of riboflavin and nicotinic acid in rats. *J Nutr* 25:173-183.
- Sauberlich HE. 1967. Biochemical alterations in thiamin deficiency-their interpretations. *Am J Clin Nutr* 20(6):528-542.
- Sauberlich HE, Dowdy RP, Skala JH. Thiamin. In: *Laboratory tests for the assessment of nutritional status*. Cleveland: CRC Press Inc., 1974.
- Schanler J. Water-soluble vitamins. Tsang LL, Nichols BL eds. In: *Nutrition in infancy* Philadelphia: Manley & Belfus Inc, 1988.
- Scheiner JM, Araiyo MM, DeRitter E. 1981. Thiamine destruction by sodium bisulphite in infusion solutions. *Am J Hosp Pharm* 38(12):1911-1913.
- Schneidlin S, Lee A, Griffith I. 1952. The action of riboflavin on folic acid. *J Am Pharmacol Assoc (Sci ed)* 41:420-427.
- Schultz AS, Light RF, Frey CN. 1938. Vitamin B₁ metabolism in man. Excretion of B₁ in urine and feces. *Proc Soc Exper Biol Med* 38:404-406.
- Selhub J and Rosenberg IH. Assessment of vitamin depletion. In: *Nutritional assessment*. Ed: Wright RA, Heyesfield S. Boston: Blackwell Scientific Publications Inc., 1984.

- Shah JJ. Riboflavin. In: Methods of vitamin assay. Ed: Augustin J, Klein BP, Becker D, Venugopal PB. Toronto: Wiley and sons, 1985.
- Shenkin A. 1986. Vitamin and essential trace element recommendations during intravenous nutrition: theory and practice. Proc Nutr Soc 45:383-390.
- Shinton NK. 1972. Vitamin B₁₂ and folate metabolism. Br Med J 1:556-559.
- Shojania AM and Hornaday G. 1970. Folate metabolism in newborns and during early infancy. I. Absorption of pteroylglutamic (folic) acid in newborns. Pediatr Res 4:412-421.
- SimulTRAC-S Solid Phase Radioassay Kit - Vitamin B₁₂ [⁵⁷Co] Folate [¹²⁵I]. Becton Dickinson Immunodiagnostics, 1982.
- Sisson TRC. 1987. Photodegradation of riboflavin in neonates. Fed Proc 46:1883-1885.
- Smith AM, Picciano MF, Peering RH. 1983. Folate supplementation during lactation: maternal folate status, human milk folate content, and their relationship to infant folate status. J Pediatr Gastroenterol Nutr 2:622-628.
- Smith JL, Canham JE, Wells PA. 1988. Effect of phototherapy light, sodium bisulfite, and pH on vitamin stability in total parenteral nutrition admixtures. JPEN 12(4):394-402.
- Smith AM, Picciano MF, Deering RH. 1985. Folate intake and blood concentrations of term infants. Am J Clin Nutr 41:590-598.
- Spector L and Lorenzo AV. 1975. Folate transport by the choroid plexus in vitro. Science 187(4176):540-21.
- Spector R. 1980. Riboflavin transport in the central nervous system. J Clin Invest 66:821-831.
- Stearns G, Adamson L, McKinley JB. 1958. Excretion of thiamine and riboflavin in children. Am J Dis Child 95:185.
- Steier M, Lopez R, Cooperman JM. 1976. Riboflavin deficiency in infants and children with heart disease. Am Heart J 92(2):139-143.
- Stephenson JM, Da JN, Oliver TK. 1970. The effect of cooling

- on blood gas tension in newborn infants. J Pediatr 76:848-852.
- Stevens D, Burman D, Strelling K, et al. 1979. Folic acid supplementation in low birthweight infants. Pediatr 64(3):333-335.
- Strelling MK, Blackledge DG, Goodall HB. 1979. The diagnosis and management of folate deficiency in low birthweight infants. Arch Dis Child 54:271.
- Strelling MK, Blackledge GD, Goodall HB et al. 1966. Megaloblastic anemia and whole blood folate levels in premature infants. Lancet 1:898-900.
- Tamura T, Yoshimura Y, Arakawa T. 1980. Human milk folate and folate status in lactating mothers and their infants. Am J Clin Nutr 33:193-197.
- Tansey RP and Schneller GH. 1955. Studies on the stabilization of folic acid in liquid pharmaceutical preparations. J Am Pharmacol Assoc (Sci ed) 44:34-37.
- Tillotson JA and Bashor. 1980. Fluorometric apoprotein titration of urinary riboflavin. Anal Biochem 107:214-219.
- Tillotson JA and Baker. 1972. An enzymatic measurement of the riboflavin status in man. Am J Clin Nutr 25:425-431.
- Tsang RL. Determining the vitamin and mineral requirements in preterm infants: An introduction. In: Vitamin and mineral requirements in preterm infants. Ed: Tsang RL. New York: Dekker, 1988.
- Tuck S. Fluid and electrolyte balance in the neonate. In Textbook of neonatology. Ed. Robertson NRL. New York: Churchill Livingstone, 1986.
- Vaz Pinto A, Lorras V, Aandoval JFF et al. 1975. Folic acid and vitamin B₁₂ determination in fetal liver. Am J Clin Nutr 28:1085-1086.
- Wilkinson JL and Cooke RWI. Cardiovascular disorders. In: Textbook of neonatology. Ed: Robertson NRC. New York: Churchill Livingstone, 1986.
- Yu VYH. Parenteral nutrition in the newborn. In: Textbook of neonatology. Ed: Robertson NRC. New York: Churchill Livingstone, 1986.
- Zlotkin SH, Bryson MH, Anderson GH. 1981. Intravenous

nitrogen and energy intakes required to duplicate in utero nitrogen accretion in prematurely low birthweight infants. J Pediatr 99:115.

Zlotkin SH and Buchanan BE. 1983. Meeting zinc and copper intake requirements of parenterally fed preterm and full term infants. J Pediatr 103:441.

Zuelzer WW and Ogden FN. 1946. Megaloblastic anemia in infancy. A common syndrome responding specifically to folic acid therapy. Am J Dis Child 71:211-243.

Zuelzer WW and Rutzky J. 1953. Megaloblastic anemia of infancy. Adv Pediatr 6:243-396.

APPENDIX B

Water Soluble Vitamin Status of Low Birthweight Infants

Water soluble vitamins are essential for life and are present in small amounts in the body and food. However, we do not know exactly what levels are required by newborn babies who are being totally fed by liquid into a vein. If you agree to allow your baby to participate, we will need:

1. A 1 ml blood sample taken once a week for a period of four weeks. These samples will be taken at the same time as routine collection.
2. At different times during the study, collections of urine which would normally be discarded. These collections will be taken during a 24 hour period which, in our experience, rarely causes irritation to the child.

There will be no known risk to your baby in the study. Although there will not be any direct benefit to your child from this study, the knowledge gained will be valuable in assisting us in our understanding of vitamin requirements during intravenous feeding for these babies.

You may decide not to participate or may withdraw from the study at any time without affecting your normal treatment.

I, _____ agree to allow my child _____ to take part in the study Water Soluble Vitamin Status of Low Birthweight Infants. I also acknowledge that the study has been made as clear to me as possible by Dr. _____ or his research assistant. Any questions have been answered to my satisfaction.

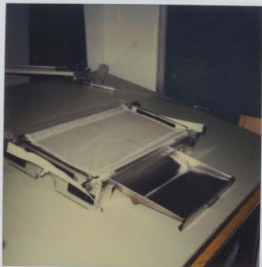
Signed _____ (Patient)

_____ (Doctor or Research Assistant)

_____ (Witness)

Date _____

APPENDIX C



"METABOLIC BED"

APPENDIX D



"SWEDISH NITEX MESH"

APPENDIX E

ERYTHROCYTE TRANSKETOLASE ACTIVITY ASSAY

SAMPLE PREPARATION

The samples were prepared by using 200 μ L of red blood cells diluted 1:1 with distilled water.

REAGENT PREPARATION

(i) Buffer:	0.9% NaCl	3.13 mL
	1.15% KCl	80.50 mL
	1.75% K_2HPO_4	15.60 mL
	3.82% $MgSO_4 \cdot 7H_2O$	0.77 mL

1 N HCl was used to bring the pH to 7.4

- (ii) TPP
 - a) stock solution: 25 mg Cocarboxylase (TPP): 25 mL buffer
 - b) working solution: 1 mL stock solution: 16 mL buffer
- (iii) 5% TCA
- (iv) Pentose Standard Solution
 - a) stock solution: 1 mg D-ribose: 1 mL distilled water
 - b) working solution: 1 mL stock solution: 100 mL distilled water (10 μ g/mL)
- (v) Orcinol reagent
 - a) "30%" HCl: 3 parts HCl conc. to 1 part distilled water
 - b) 2 g orcinol } to 50 mL water
 - 0.165 g $FeCl_3 \cdot H_2O$ }
 - c) dilute b) to 1000 mL with "30%" HCl
- (vi) Substrate: 0.084 g Ribose-5-phosphate disodium salt to 7 mL distilled water. Add buffer to 6.8 mL substrate to obtain a volume of 10 mL.

CALCULATIONS

The amount of pentose utilized per mL hemolysate per hour is calculated following the steps outlined below.

- (i) Dilution Factor (DF)

$$DF = \frac{1}{.1^b} \times \frac{3.6^a}{1} \times \frac{1}{.2^c} = 180$$

^aamount of hemolysate

^btotal volume

^cmL filtrate used

- (ii) Average absorbance/ μ g pentose standard (SP)

$$SP = (OD_5/5 + OD_{10}/10) \div 2$$

- (iii) $KP = DF/SP$

- (iv) $2R + D$ = amount of pentose originally present in the group of tubes prior to incubation

- (v) $TP_1 = (2R + D - A) \times KP = \mu\text{g pentose used/mL hemolysate/h without TPP}$

- (vi) $TP_2 = (2R + D - B) \times KP = \mu\text{g pentose used/mL hemolysate/h with TPP}$

- (v) $TPPE\% = TP_2 - TP_1/TP_1 \times 100$

For contents of tubes A, B, R and D refer to Tables 5.1 and 5.2.

A = absorbance without TPP

B = absorbance with TPP

D = amount of pentose endogenous to sample

R = this tube contains substrate to determine the amount of pentose utilized

APPENDIX F

ERYTHROCYTE GLUTATHIONE REDUCTASE ACTIVITY ASSAY

SAMPLE PREPARATION

The samples were prepared by using 50 μ L red blood cells diluted with 950 μ L distilled water.

REAGENT PREPARATION

All reagents except the buffer were prepared the day of the assay and kept on ice.

- (i) 0.27 mM FAD: 0.0022 g/10 mL distilled water
- (ii) 1.9 mM NADPH: 0.016 g/10 mL 1% sodium bicarbonate solution
- (iii) 7.4 mM GSSG: 0.0452 g/10 mL distilled water/0.1 mL 1 N NaOH
- (iv) 80 mM EDTA (tetrasodium salt): 0.1521 g/5 mL distilled water
- (v) 0.1 M potassium phosphate buffer (pH brought up to 7.4 with 5N NaOH)

CALCULATIONS

ACTIVITY COEFFICIENT (AC) = $\frac{\text{REDUCTION OF ABSORBANCE WITH FAD}}{\text{REDUCTION OF ABSORBANCE WITHOUT FAD}}$

APPENDIX G

RADIOASSAY PROCEDURE-FOLATE AND VITAMIN B₁₂ DETERMINATION

SAMPLE PREPARATION

- (i) PLASMA FOLATE AND VITAMIN B₁₂: The samples were diluted to a 1:1 ratio with distilled water.
- (ii) RBC FOLATE: Fifty microlitres (50 µL) of whole blood was diluted with 1 mL of freshly prepared 0.2% ascorbic acid solution (w/v) (1:21 dilution). The hematocrit is necessary for the determination of RBC folate.

CALCULATIONS

1) PLASMA FOLATE AND VITAMIN B₁₂

- Average the counts found for the blank tubes (3 & 4) and subtract this value from all other tubes to obtain corrected counts
- Average the total counts (1 & 2) to obtain the Total Count per assay
- Trace Binding, B₀ (should be > 35%)
$$B_0 = \frac{\text{average corrected counts (5 \& 6)}}{\text{Total Count (1 \& 2)}} \times 100$$
- % Trace Binding = $\frac{\text{corrected count (samples)}}{\text{average corrected counts (5 \& 6)}} \times 100$
- Plot standard curve on logit-log paper (Appendix H).
% Trace Binding vs pg/mL vitamin B₁₂ or ng/mL folate.
- Concentration of vitamin B₁₂ or folate in samples can be obtained from the standard curve and multiplied by the dilution factor.

2) RBC FOLATE

- Calculate % Trace Binding for the hemolysate
- Obtain the concentration of folate from the standard curve
- ng/mL of packed red cells = $\frac{(\text{ng/mL hemolysate}) \times 21}{\% \text{ hematocrit}/100}$

The image shows a document page that is extremely degraded. It features a grid-like structure, likely a table or form, with numerous columns and rows. The text within these cells is mostly lost due to heavy noise and high contrast. A significant diagonal artifact, possibly a fold or a tear, runs across the lower right quadrant of the page. The overall appearance is that of a corrupted scan of a historical or official document.

APPENDIX I

URINARY THIAMIN ASSAY (THIOCHROME PROCEDURE)

REAGENT PREPARATION

The reagents were prepared the day of the assay.

- (i) 1 N NaOH
- (ii) Isobutanol
- (iii) Oxidizing Reagent: 2 parts 2% potassium ferricyanide
to 4 parts 40% NaOH
- (iv) Benzenesulphonyl chloride (BSC)
- (v) 3% H₂O₂
- (vi) Standard thiamin solution (0.2 µg/mL)

CALCULATIONS

$$\mu\text{g thiamin/mL urine} = \frac{\text{Tube B} - \text{Tube C}}{\text{Tube A} - \text{Tube B}} \times \frac{0.4}{2}$$

Tube B - Tube C represents the fluorescence due to thiochrome produced from thiamin in urine.

Tube A - Tube B represents the fluorescence due to the thiochrome produced from the added thiamin standard.

APPENDIX J

URINARY RIBOFLAVIN ASSAY (FLUOROMETRIC APOPROTEIN TITRATION)

SAMPLE PREPARATION

Dilute control urine 1:10 or 1:100 with TRIS and subjects urine 1:200. Standards were prepared with TRIS and ranged from 0 -40 ng/mL.

REAGENT PREPARATION

- (i) 0.05 M TRIS, pH 7.5 (concentrated HCl was used to obtain desired pH)
- (ii) Standard riboflavin solution
 - a) Stock solution: Dissolve 19 mg of riboflavin up to 500 mL with distilled water which contains 1 mL HCl (0.038 mg/mL)
 - b) Working solution: Dilute 1 mL stock solution up to 200 mL with distilled water (190 ng/mL)
- (iii) Apoprotein: Dissolve 1 mg apoprotein with 1 mL 0.05 M TRIS.
Dilute 1:4 with TRIS and keep on ice.

APPENDIX K

STATISTICAL FORMULA (DESIGNED BY DR. SUTRADHAR, DEPARTMENT OF MATHEMATICS AND STATISTICS, MUN).

EXPERIMENTAL DESIGN

	T_1	T_2	T_3	
U_1	n_{11}, \bar{x}_{11}	n_{21}, \bar{x}_{21}	n_{31}, \bar{x}_{31}	N_{01}
U_2	n_{12}, \bar{x}_{12}	n_{22}, \bar{x}_{22}	n_{32}, \bar{x}_{32}	N_{02}
U_3	n_{13}, \bar{x}_{13}	n_{23}, \bar{x}_{23}	n_{33}, \bar{x}_{33}	N_{03}
	N_{T1}	N_{T2}	N_{T3}	

$$H_0: T_1 = T_2 = T_3$$

$$H_0: U_1 = U_2 = U_3$$

where T = treatment
 U = time period
 N_T = total sample for treatment
 N_0 = total sample at each time period
 n = sample size per cell
 \bar{x} = mean per cell

- A) To determine if there is a treatment effect between groups, calculate a 5% confidence interval using the following procedure:

Example: for $T_1 - T_2$

$$(\hat{T}_1 - \hat{T}_2) - 1.96 \sqrt{V(\hat{T}_1 - \hat{T}_2)} < T_1 - T_2 < (\hat{T}_1 - \hat{T}_2) + 1.96 \sqrt{V(\hat{T}_1 - \hat{T}_2)}$$

$$\text{where; } \hat{T}_1 = \frac{n_{11}\bar{X}_{11} + n_{12}\bar{X}_{12} + n_{13}\bar{X}_{13}}{N_{T1}}$$

$$\hat{T}_2 = \frac{n_{21}\bar{X}_{21} + n_{22}\bar{X}_{22} + n_{23}\bar{X}_{23}}{N_{T2}}$$

$$V(\hat{T}_1 - \hat{T}_2) = V(\hat{T}_1) + V(\hat{T}_2) - 2 \text{Cov}(\hat{T}_1, \hat{T}_2)$$

$$\therefore 2 \text{Cov}(\hat{T}_1 - \hat{T}_2) = 0$$

$$\therefore V(\hat{T}_1 - \hat{T}_2) = V(\hat{T}_1) + V(\hat{T}_2)$$

$$\text{where } V(\hat{T}_1) = \frac{1}{N_{T1}^2} \left\{ n_{11}\sigma_{11}^2 + n_{12}\sigma_{12}^2 + n_{13}\sigma_{13}^2 + 2(n_{11}n_{12}\sigma_{12} + n_{11}n_{13}\sigma_{13}) \right. \\ \left. + n_{12}n_{13}\sigma_{23} \right\},$$

$$\text{where } \sigma(\text{covariance}) = \frac{1}{n-1} \sum_{i=1}^n (x_i - \bar{x})(y_i - \bar{y})$$

*A random sample was obtained for each time period using the smallest n for treatment and assuming a homogenous group within the cells.

$$V(\hat{T}_2) = \frac{1}{N_{T2}^2} \left\{ n_{21}\sigma_{21}^2 + n_{22}\sigma_{22}^2 + n_{23}\sigma_{23}^2 + 2(n_{21}n_{22}\sigma_{12} + n_{21}n_{23}\sigma_{13}) \right. \\ \left. + n_{22}n_{23}\sigma_{23} \right\}$$

B) To determine if there is a time effect within groups, calculate a 5% confidence interval using the following procedure:

Example: for $\hat{U}_1 - \hat{U}_2$

$$(\hat{U}_1 - \hat{U}_2) - 1.96 \sqrt{V(\hat{U}_1 - \hat{U}_2)} < U_1 - U_2 < (\hat{U}_1 - \hat{U}_2) + 1.96 \sqrt{V(\hat{U}_1 - \hat{U}_2)}$$

$$\text{where; } \hat{U}_1 = \frac{n_{11}\bar{X}_{11} + n_{21}\bar{X}_{21} + n_{31}\bar{X}_{31}}{N_{U1}}$$

$$\hat{U}_2 = \frac{n_{12}\bar{X}_{12} + n_{22}\bar{X}_{22} + n_{32}\bar{X}_{32}}{N_{U2}}$$

$$V(\hat{U}_1 - \hat{U}_2) = V(\hat{U}_1) + V(\hat{U}_2)$$

$$V(\hat{U}_1) = \frac{1}{N_{U1}^2} \left\{ n_{11}\sigma_{11}^2 + n_{21}\sigma_{21}^2 + n_{31}\sigma_{31}^2 + 2(n_{11}n_{21}\sigma_{12} + n_{11}n_{31}\sigma_{13}) \right. \\ \left. + n_{21}n_{31}\sigma_{23} \right\},$$

$$\therefore 2 (n_{11}n_{21}\sigma_{12} + n_{11}n_{31}\sigma_{13} + n_{21}n_{31}\sigma_{23}) = 0$$

$$\therefore V(\hat{U}_1) = \frac{1}{N_{U1}^2} (n_{11}\sigma_{11}^2 + n_{21}\sigma_{21}^2 + n_{31}\sigma_{31}^2)$$

$$V(\hat{U}_2) = \frac{1}{N_{U2}^2} (n_{12}\sigma_{12}^2 + n_{22}\sigma_{22}^2 + n_{32}\sigma_{32}^2)$$



